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DIISOPROPYL PHOSPHOFLUORIDATE-INDUCED ANTINOCICEPTION

by

GARY L. KOEHN

A Dissertation Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

March

1980

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## ACKNOWLEDGEMENTS

This dissertation is the product of a continuing research and educational process which involves many individuals who have contributed to its development and completion. It is a pleasure to acknowledge the contributions of these individuals.

I am most grateful to my advisor, Dr. A.G. Karczmar, for his suggestions, encouragement, and patience throughout my graduate career.

I would also like to thank Dr. G. Henderson for his suggestions and help concerning the enkephalin bioassay and related studies. The efforts of the other committee members, Drs. E. Anderson, E. Moon, and R.A. North are gratefully acknowledged.

The invaluable assistance of Michael Avram, Lionel Barnes, Ann Ingerson, Gisela Kindel, Joan Sim, and John Williams is acknowledged.

Additionally, the following generous donations are acknowledged: (i) naloxone HCl, Dr. A. Rubin, Endo Laboratories; (ii) GPA 1843 and GPA 1847, Dr. F.H. Clark, Ciba-Geigy Corporation; (iii) MR 2266 and MR 2267, Boehringer Ingelheim Company (through Dr. R.A. North); and (iv) d- and l-cyclazocine, Mr. A.E. Soria, Sterling-Winthrop Research Institute.

Finally, I would like to thank my wife, Sharon, and my parents

for their continued support and unselfish love which has meant more than anything else to me.

## VITA

The author, Gary Lee Koehn, is the son of Paul Frederick and Dorothy (Ornellos) Koehn. He was born August 18, 1955, in Oak Park, Illinois.

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Koehn, G.L. and Karczmar, A.G. (1978). Effects of diisopropyl phosphofluoridate on analgesia and motor behavior in the rat. *Prog. Neuro-Psychopharmacol.* 2, 169-177.

Barnes, L., Koehn, G.L. and Karczmar, A.G. (1978). Effects of diisopropyl phosphofluoridate (DFP) on pain threshold and serotonin. *Seventh Inter. Cong. Pharmacol. Abstr.* 204.

Koehn, G.L., Henderson, G. and Karczmar, A.G. (1979). Diisopropyl phosphofluoridate-induced antinociception in rats: Possible role of endogenous opioids. *Fed. Proc. Abstr.* 39, 363.

Koehn, G.L., Henderson, G. and Karczmar, A.G. (1980). Diisopropyl phosphofluoridate-induced antinociception in rats: Possible role of endogenous opioids. *Eur. J. Pharmacol.* 61, 167-175.

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## 1. REVIEW OF THE RELATED LITERATURE

### 1.1 Pain Mechanisms

#### 1.1.1 Historical Account

Pain is one of the most common experiences of man and the explanations of its nature probably one of his oldest preoccupations. Throughout the years, several theories have been proposed to explain the phenomenon of pain; as research evidence began to accumulate all these theories have been shown to be inadequate.

One of the earlier writers on pain, Aristotle, considered pain to be a manifestation of the soul, an emotion, and the epitome of unpleasantness (Aristotle [330 B.C.?] cited by Hardy et al., 1952). Man was thought to continuously fluctuate between two emotional states of pleasure and pain; thus, pain was equated with the unpleasantness one experiences throughout his/her life (Aristotle [330 B.C.?] cited by Hardy et al., 1952).

The view of pain as an emotional state was championed until the end of the eighteenth century when Darwin proposed the Intensive Theory of pain (Darwin, 1794, cited by Hardy et al., 1952). The Intensive Theory considered pain to occur whenever any of the "sensorial emotions" namely heat, touch, sight, taste, or smell were stronger than usual (Darwin, 1794, cited by Hardy et al., 1952). Although the Intensive Theory was popular for a long time, evidence now demon-

strates that in some cases pain is in itself a particular form of sensation (Sweet, 1959; Dash and Deshpande, 1976).

The twentieth century saw the Intensive Theory of pain replaced by the Specificity Theory which until recently was the theory taught in medical schools (DeSousa and Wallace, 1977). The Specificity Theory implies a straight-through transmission system from somatic pain receptors via specialized pain fibers and a pain pathway to a "pain center" in the brain (Sweet, 1959). Evidence today, however, suggests that the perception of pain cannot be directly related to the neural activity in any particular anatomical structure (Melzack and Wall, 1965). Furthermore, pain is influenced by many psychological variables such as attention, anxiety, suggestion, and prior conditioning; thus, the perception of pain includes a very pervasive psychological component (Melzack and Wall, 1965).

Recently, research concerning pain has moved in the direction of investigating the plasticity and modifiability of events in the central nervous system (CNS) through various physiological and psychological techniques; these techniques will be reviewed briefly; pertinent physiological and anatomical descriptions also will be presented. In addition, the involvement of the cholinergic, serotonergic, and endogenous opioid systems in the phenomenon of pain will be reviewed. However, before proceeding any further, it is necessary to define some relevant terms which will be employed throughout this dissertation.

### 1.1.2 Definitions

Just as there have been many theories proposed to explain the phenomenon of pain, many definitions have been proposed. Analysis of the current literature suggests that the term "pain" must be defined in terms of psychology rather than physiology, and therefore is only applicable for use when describing the human experience. Merskey (1978) has proposed the following definition of pain: "pain is an unpleasant experience which we primarily associate with tissue damage or describe in terms of tissue damage or both".

Next, the term "nociception" is defined as potentially tissue-damaging thermal, mechanical, or chemical energy impinging upon specialized nerve endings called nociceptors (Fordyce, 1978). In this scheme, the pain experience must be perceived in order for it to be said to have occurred. However, nociceptive input for pain needs not occur since man may perceive pain even in the absence of noxious stimuli such as in phantom limb pain. Similarly, the presence of noxious stimuli does not insure the experience of pain since various neurological defects, the administration of drugs, or other factors make it possible for noxious stimuli to exist without being perceived. Thus, the terms pain and nociception although related to one another clearly are not interchangeable.

Finally, the terms "analgesia" and "antinociception" are defined as the failure of an organism to exhibit pain- and nociceptive-induced behaviors, respectively; behavior is defined as observable, measurable,

overt, verbal and non-verbal actions of an organism (Fordyce, 1978). In regards to pain and nociception, the essence of the problem is that there are pain- and nociceptive-induced behaviors. It is these particular behaviors such as the various autonomic responses and voluntary or reflex muscular movements in laboratory animals as well as the verbal and non-verbal descriptions by humans which are observed and quantitated in the study of nociception and pain.

### 1.1.3 Psychological Factors Influencing Pain

Presently, there exists a large and increasing body of evidence which suggests that pain is influenced by a variety of psychological factors (Sternbach, 1978). To understand pain first one must take into account its interaction with the overall total personality. Since an individual's personality is shaped from birth, a developmental analysis might prove useful in demonstrating the importance of early childhood events in shaping the pain experience. Pozanski (1976) demonstrated that children (age 5 to 16 years) who exhibit recurrent pain syndromes, for which there is no evident organic causation, such as chronic abdominal pain, headache, and limb pain come from families in which the parents themselves were more prone to exhibit pain behaviors. Pozanski (1976) suggests that the models presented by parents influenced pain associated behaviors in their children.

Second, pain also has a very important cultural dimension. People respond to pain not only as individuals but also as members of

their cultural heritage. While differences in pain behavior among cultural and ethnic groups are not well understood, consistencies within a particular group suggest that members of the group model normative standards for both the degree and way in which suffering is perceived and exhibited (Craig, 1978). Cultural specific attitudes towards pain can be summarized as follows: Yankees are apathetic, matter of fact, doctor-help oriented; Jews are concerned about implications of pain and distrust pain relief procedures; Italians readily express desire for pain relief; Irish block both expressions of suffering and concern for the implications of pain (Craig, 1978).

Third, some reinforcing, environmentally situated events may be pain contingent; that is, certain reinforcing events will not occur unless preceded by pain behaviors. Pain medications prescribed on a "take only as needed" basis may be effective reinforcers or positive consequences of pain-related behavior or pain perception if the patient feels better when sedated or analgetic (Fordyce, 1978). Similarly, accident victims or wounded war heroes who are in pain may receive love, attention, and affection as rewards for pain behavior (DeSousa and Wallace, 1977). Finally, pain behavior is often rewarded financially; monetary compensation after successful litigation is an excellent reinforcement causing people to remain in pain (DeSousa and Wallace, 1977).

Indirect reinforcement of pain behavior may occur when pain behavior leads to avoidance of some aversive or unpleasant consequence such as avoiding an unpleasant job, a threatening social encounter,

or an aversive set of duties and responsibilities (Fordyce, 1978). Paraphrased, "when I am in pain, bad things don't happen which otherwise would (Fordyce, 1978)".

Fourth, in order for any stimulus to produce pain it must affect either the arousal or selective aspects of the patient's attention. Since an individual possesses a finite attention capacity, it follows that involvement of attention in one area will be accompanied by removal of attention from another area. In fact, almost any situation that attracts intensive maintained attention will diminish or abolish pain perception (Melzack, 1961).

Strong relationships between pain and mood have been demonstrated. Anxiety is generally recognized as increasing pain; thus, anything which diminishes anxiety may be expected to diminish pain (Merskey, 1978). Even the mere presence of the word pain during instructions in an experimental situation decreased the level of electroshock pain in patients as compared to a group of patients in which the mention of pain was not made during the instructional process (Hall and Stride, 1954). On the other hand, excitement or aggression may leave subjects totally oblivious even to serious trauma. Most notable examples are (i) football players and other sportsmen who suffer traumatic blows during competition but do not notice any pain until the contest is completed and (ii) severely wounded soldiers performing heroic tasks in spite of apparently incapacitating injuries (Merskey, 1978).

Overall, a most important psychological component of pain has

been amply demonstrated. The next section reviews the many pertinent physiological and anatomical aspects of pain.

#### 1.1.4 Physiological and Anatomical Correlates

##### 1.1.4.1 Nociceptors

Specific somatic receptors, called nociceptors, which convey neural impulses arising from a noxious stimuli have been identified and characterized as follows: (i) nociceptors are much more resistant to damage from noxious input than the low threshold mechanical and thermal receptors (ii) nociceptors have higher thresholds with respect to all stimuli in comparison to other sensory receptors of the same tissue and (iii) unlike other receptors, nociceptors undergo the process of sensitization; that is, repeated stimulation reduces the threshold for activation and increases the frequency of discharge per unit of stimulus (Perl, 1976).

Nociceptors can be classified by measuring the discharge frequency of isolated peripheral nerves in response to a variety of noxious and innocuous stimuli applied to the skin (Besson and Perl, 1969). Briefly, (i) "mechanical nociceptors" respond to strong mechanical stimuli but are not at all excited by noxious thermal stimuli, (ii) "mechanical and thermal nociceptors" respond to both strong mechanical and noxious cold, and (iii) "polymodal nociceptors" respond to noxious mechanical, cold, and heat stimuli (Angel, 1977). In addition, a fourth group of nociceptors called "chemosensitive nociceptors" has

been identified in the anesthetized skin of leprosy patients; the application of several algescic substances to blistered skin produced pain as measured by the patients verbal reports; noxious thermal and mechanical stimuli were ineffective (Dash and Deshpande, 1976).

The morphological structure of nociceptors has not been identified unequivocally. Attempts to describe the morphological substrate for nociceptors have been based upon delineation of the receptor function of a spot of skin with subsequent fixation, excision, and histological examination, or by the functional isolation of a known structure and accurate determination of its adequate stimulus (Angel, 1977). Research in this area has been concentrated on the receptor structure in the human tooth since most authorities believe that only the sensation of pain is felt when any form of stimulation is applied to the dental pulp (Scott and Maziarz, 1976).

For many years, free nerve endings were considered to be nociceptors. However, most receptors in mammalian hairy skin are of this type indicating that such endings probably subserve sensations other than pain (Weddell et al., 1955). In addition, the cornea is innervated exclusively by free nerve endings but mediates sensations of touch, pressure, and temperature as well as pain (Lale and Weddell, 1956). Although no morphological differences are seen among free nerve endings, the possibility of physiological specificity remains.



#### 1.1.4.2 Primary Afferent Nerve Fibers

Nerve impulses generated by stimulation of nociceptors in the body tissues are transmitted along primary afferent nerve fibers to various destinations in both the spinal cord and brain (Melzack, 1973). The peripheral nerve contains a heterogeneous mixture of primary afferent fiber types which can be classified by utilizing a knowledge of nerve fiber diameter and impulse conduction velocity. A simple classification scheme is summarized as follows: the "A" fiber group consists of five subgroups of myelinated fibers designated in order of diminishing fiber diameter (20 to 1  $\mu\text{m}$ ) from alpha ( $\alpha$ ) through epsilon ( $\epsilon$ ); the conduction rate of nerve impulses (120 to 4 m/s) varies directly with the diameter of the axon (Angel, 1977). "C" fibers are unmyelinated, have diameters of 0.3 to 1.5  $\mu\text{m}$ , and conduction rates of 0.4 to 2.0 m/s (Angel, 1977).

Clark et al. (1935) demonstrated that for the cat C fibers conducted to the CNS neural impulses which elicited autonomic responses similar to those produced by noxious stimulation; A fibers were ineffective.

In humans, electrical stimulation of A  $\alpha$  fibers has been correlated with pricking pain sensations whereas stimulation of A-delta ( $\delta$ ) and C fibers produced prolonged, chronic, dull, sometimes burning pain sensations (Torebjork, 1974; Torebjork and Hallin, 1974; Willer et al., 1978).

Clinical observations do not completely support the role played by these small diameter primary afferent fibers in pain perception; for instance, two peripheral neuropathies, namely Fabry's Disease and a hereditary sensory disease both result in a decrease of the number of small diameter primary afferent fibers found in the peripheral nerve, but are associated with increased and decreased pain sensations, respectively (Wall, 1978). However, since pain perception involves numerous higher brain centers as well as primary afferent fibers it would be difficult to speculate about the changed state of pain perception in neuropathies on the basis solely of the state of the primary afferent nerve fiber.

Finally, Applebaum et al. (1976) demonstrated that 50% of the small unmyelinated fibers in the ventral roots arise from dorsal root ganglion and are sensory fibers. Furthermore, many of these fibers are selectively responsive to noxious cutaneous mechanical and thermal stimuli (Clifton et al., 1976). Thus, these fibers may also participate in the transmission of neural impulses arising from noxious stimulation.

#### 1.1.4.3 Spinal Cord Involvement

The cytoarchitectonic investigations of Rexed (1952) established that neurons of the dorsal horn of the spinal cord are arranged in a series of six clearly defined laminae. The surface of each lamina is roughly parallel to the dorsal and ventral surfaces of the cord; laminae are numbered consecutively I through VI from the extreme

dorsal aspect to the center of the spinal cord.

Christensen and Perl (1970) demonstrated that lamina I neurons are excited by intense thermal and mechanical cutaneous stimulation. This finding has been confirmed in several laboratory animals including the rat (Giesler et al., 1976). Although experiments involving recording from lamina II cells have not been performed extensively, Perl (1976) has shown that some lamina II cells were excited by high intensity thermal and mechanical stimuli.

Neurons in lamina V can be excited by noxious thermal and mechanical stimuli (Price and Mayer, 1975). These neurons in contrast to lamina I neurons also respond to innocuous thermal and mechanical stimuli; their firing rates increase as the stimulus increases throughout a range of innocuous to noxious intensities; these neurons have been called "wide dynamic range neurons" (Price and Mayer, 1975; Giesler et al., 1976).

Neurons located in laminae III and IV show excitatory responses to innocuous touch and pressure movements and pressure stimulation; however, these neurons were not excited by noxious pressure stimulation (Giesler et al., 1976).

In summary, it appears that there are two populations of neurons in the dorsal horn which are responsive to noxious stimulation: (i) lamina I, marginal, neurons and (ii) lamina V, wide dynamic range neurons.

#### 1.1.4.4 Ascending Transmission Systems

Three anatomically distinct pathways convey sensory information from the periphery to the cerebral cortex; they include (i) the dorsal column-lemniscothalamic system, (ii) the spinocervical-lemniscothalamic system, and (iii) the spinothalamic system which consists of spinoreticular, paleospinothalamic, and neospinothalamic components (Angel, 1977). It appears that all three ascending transmission systems participate in the transmission of neural impulses arising from noxious input (Dennis and Melzack, 1977).

For years the dorsal columns has been viewed as carriers only of innocuous touch and proprioceptive information. While this still appears to be the case for primary afferent fibers ascending in the dorsal columns it can no longer be said of the secondary afferent fibers. Petit (1972) determined that 9.3% of fibers examined in the dorsal columns originated from spinal neurons. The frequency of evoked tonic discharge in these secondary afferent fibers increased accordingly to the strength of applied heat and mechanical stimulus even when the stimulus was brought well into the noxious range (Petit, 1972). Thus, the dorsal column-lemniscothalamic system has the capacity to transmit nociceptive information.

Neurons which demonstrate increased discharge frequencies in response to noxious mechanical and thermal stimuli have been shown to be present in the spinocervical tracts of the cat (Price and Brown, 1975) and monkey (Bryan et al., 1974). In addition, some of these

cells respond exclusively to noxious mechanical stimulation (Bryan et al., 1974).

The spinothalamic system is believed to be the principal pathway for conveying nociceptive information in man and primates (Willis, 1976). Trevino et al. (1974) demonstrated that many spinothalamic neurons have their origins in spinal cord laminae known to be associated with the transmission of nociceptive information such as laminae I, IV, and V (see section 1.1.4.3).

Price and Mayer (1975) demonstrated that 50% (41 out of 82) of the cells studied in the anterolateral quadrant of the spinal cord responded to both innocuous and noxious mechanical stimuli while 12% (10 out of 82) of the cells studied responded only to noxious mechanical stimuli. Similar results in studies of the projections to various midline and intra-laminar thalamic nuclei (Willis, 1976) via the spinothalamic tract as well as to the medullary reticular formation (Fields et al., 1975) via the spinoreticular tract also have been obtained.

Finally, it should be pointed out that specific central structures (such as the nuclei of the lateral diencephalon) which receive neural input via the rapidly conducting dorsal column, spinocervical, and neospinothalamic tracts are activated prior to other central sites (such as midline and intralaminar thalamic nuclei and brain stem reticular formation) to which the slowly conducting paleospinothalamic and spinoreticular tracts ascend, respectively

(Bowsher, 1976). Dennis and Melzack (1977) suggest that the rapidly conducting systems convey phasic information about pain whereas the slowly conducting systems carry tonic information.

Overall, it appears that all of the ascending tracts discussed above participate in the transmission of nociceptive information to various higher centers in the CNS. The heterogeneity of the ascending systems in terms of termination sites as well as the rate at which neural impulses are transmitted suggest that specific ascending systems may selectively activate not only anatomically specific brain sites but also physiologically specific mechanisms.

#### 1.1.4.5 Supraspinal Associated Structures

Various supraspinal structures such as the reticular formation, thalamus, and cortex have been implicated in processing information concerning noxious stimulation (Kerr and Wilson, 1978).

Casey et al. (1974) have demonstrated that 57% (59 out of 104) of the units studied in the medullary nucleus reticularis gigantocellularis responded to innocuous and noxious mechanical stimuli; in addition, 25% of these units responded exclusively to noxious mechanical stimuli. Investigations of the nucleus ventralis of the medulla have also demonstrated that a majority of cells (70%) respond to noxious heat and mechanical stimulation; some of these cells respond exclusively to noxious stimulation (Benjamin, 1970).

Imprecisely defined regions in the pontine reticular formation

(Eickhoff et al., 1978), mesencephalic reticular formation (Young and Gottschaldt, 1976; Eickhoff et al., 1978), and mesencephalic central grey region (Eickhoff et al., 1978) also contain cells which respond to noxious stimulation. Again, the majority (>70%) of the units tested in each study responded to innocuous as well as noxious heat and mechanical stimuli; some (>16%) of the units tested respond exclusively to noxious stimulation.

The thalamic posterior group of nuclei (PO) contain neurons which respond to noxious stimulation of the skin; 30% of 258 neurons studied were determined to be nociceptive; 16% responded exclusively to noxious mechanical stimuli while 14% of the cells responded differentially to both noxious and innocuous mechanical stimulation (Dong and Wagman, 1976).

Similarly, neurons in the medial thalamus particularly the nucleus parafascicularis, respond to noxious mechanical stimuli; however, more than half of the nociceptive neurons in the medial thalamus, unlike the nociceptive cells in the PO, did not respond to innocuous mechanical stimulation (Dong et al., 1978). Thus, the thalamus participates in the nociceptive phenomenon.

Cerebral cortical involvement in the appreciation of nociceptive input has been suggested by the following experiments. First, ablation of the secondary somatosensory cortex, the anterior ectosylvian gyrus, and its bordering sulci increased the escape threshold to noxious electric shock in cats; ablation of the primary somatosensory cortex was

ineffective (Berkely and Parmer, 1974).

Second, specific evoked potentials (EPs) have been demonstrated to arise from noxious tooth pulp stimulation in man; tooth pulp-evoked potentials were not present when devitalized teeth of normal patients were stimulated or when an individual congenitally insensitive to pain was used as the subject (Chatrian et al., 1975). Recently, Chen et al. (1979) demonstrated that the amplitudes of specific components of the EP waveform were directly related to the stimulus intensity and subjective pain response as measured by the subject's verbal reports.

#### 1.1.4.6 Descending Systems

Anatomically, the brain stem reticular formation receives input fibers from a number of higher centers. Axon degeneration studies have demonstrated that fibers originating in all parts of the cerebral cortex, especially the motor cortex, descend to terminate in the brain stem reticular formation; the majority of these fibers end in two fairly well defined areas, the pontine nucleus pontis oralis, and the medullary nucleus reticularis gigantocellularis (Rossi and Brodal, 1956). Electrical stimulation of intralaminar and midline thalamic nuclei has been shown by intracellular recording techniques to depolarize cells located in pontine and medullary reticular formation (Mancia et al., 1974a). The existence of intrareticular connections within the brainstem itself which produce primarily ascending inhibitory effects also have been demonstrated by intracellular recording



techniques (Mancia et al., 1974b).

Neuron degeneration studies show that reticulospinal fibers from the medial portions of the pontine and medullary reticular formation descend to terminate in cervical and thoracic spinal cord; more than half of the cells in specific reticular nuclei namely pontis caudalis, oralis, gigantocellularis, ventralis, and lateralis project to the spinal cord (Torvik and Brodal, 1957).

Recently, descending reticulospinal projections from medullary reticular nuclei were studied with autoradiographic methods; the nucleus reticularis gigantocellularis projects primarily to motor related areas such as laminae VII and VIII; the nuclei raphe magnus and reticularis magnocellularis project primarily to laminae with known nociceptive input such as laminae I, II, III, and V (Basbaum et al., 1978; see section 1.1.4.3).

Overall, the brain exhibits the anatomical capacity to influence sensory input at several levels of the CNS. An analysis of how these descending systems affect nociception will be discussed in sections 1.3.4 and 1.4.4.

#### 1.1.5 Gate Control Theory

The Gate Control Theory of pain developed by Melzack and Wall in 1965 has attracted its share of criticism; nevertheless, it remains the most readily accepted theory of pain today. The basic proposals of this theory as set forth by Melzack and Wall (1965) will be pre-

sented as follows.

First, the transmission of nerve impulses by primary afferent nerve fibers to spinal cord transmission cells (T cells) is modulated by a spinal gating mechanism in the dorsal horn. The substantia gelatinosa, laminae II and III, is considered to be the site of the spinal gating mechanism.

Second, the spinal gating mechanism is influenced by the relative amounts of activity in large, A $\alpha$  fibers and small diameter A $\delta$  and C fibers; activity of large fibers tend to inhibit transmission (close the gate) while activity in the small fibers tend to facilitate transmission (open the gate). The mechanism of this effect is that large A $\alpha$  fibers have a brief excitatory effect on spinal T cells but then close the gate by inhibiting transmission from A $\alpha$ , A $\delta$ , and C fibers through activation of interneurons in the substantia gelatinosa. On the other hand, A $\delta$  and C fibers prevent this inhibitory effect by inhibiting the inhibitory interneurons, thus opening the synaptic gate and increasing excitatory input to the T cells. However, clinical observations do not completely support this hypothesis (see section 1.1.4.2).

Third, the spinal gating mechanism is influenced by nerve impulses that descend from the brain. As described previously, various cognitive factors such as attention, anxiety, etc. influence pain responses. The brain stem reticular formation as well as reticulospinal projections inhibit transmission from the spinal T cells. Since

fibers from the cortex project to the reticular formation as well as directly to the spinal cord by means of corticospinal fibers this provides a system by means of which cognitive processes are able to influence spinal T cell activity.

Fourth, a specialized system of large diameter, rapidly conducting fibers (the central control trigger) activates selective cognitive processes which then influence, by way of descending fibers, the modulatory properties of the spinal gating mechanism. The dorsal column medial lemniscal and dorsolateral systems could fulfill the function of the central control trigger (Melzack and Dennis, 1978).

The fifth proposal is that when the output of the spinal cord T cells exceeds a critical level, it activates those neuronal areas which subserve the complex, sequential pattern of pain related behavior.

A final aspect of the Gate Control Theory is referred to as the central biasing mechanism (Melzack and Dennis, 1978). Here, brainstem areas which are known to exert an inhibitory control over transmission in the pain signalling system receive inputs from widespread parts of the body and in turn project to widespread parts of the spinal cord and brain. Stimulation of the small diameter afferents can increase input to this central biasing mechanism resulting in the closing of the gates to noxious inputs from selected body areas. The cells of the midbrain reticular formation are known to have large receptive fields and electrical stimulation of reticular formation

can produce analgesia in discrete areas of the body. It is possible then, that particular body areas may project especially to some reticular areas, and these in turn would close the gate to input from particular parts of the body. This model could be used to explain aspects of hyperstimulation analgesia of which acupuncture and transcutaneous electrical stimulation are but two examples.

#### 1.1.6 Brain Mechanisms

It is obvious that the phenomenon of pain cannot be viewed solely in terms of sensory perception without regarding motivational affective and cognitive processes. Melzack and Casey (1968) proposed a three dimensional psychological scheme to include (i) sensory-discriminative, (ii) motivation-affective, and (iii) cognitive-evaluative components of pain. A brief summary of their proposal follows.

First, the neospinothalamic tract, the spinocervical tract, and postsynaptic elements in the dorsal column-medial lemniscal system all project at least in part to the ventral basal thalamus which shows a discrete somatotopic organization. Since all of these ascending systems appear to be involved in the transmission of nociceptive information, these rapidly conducting tracts have the capacity to process information concerning the spatial, temporal, and magnitude properties of the nociceptive input. Therefore, this system may subserve the sensory-discriminative dimension of pain.

Second, the spinoreticular and paleospinothalamic tracts project

to the brainstem reticular formation and limbic system. Since the reticular formation and limbic system have been shown to participate in the pain process, the powerful motivational drive and unpleasant affective characteristics of pain may be influenced by activities in those particular brain regions which are affected primarily by the slowly conducting spinal systems.

Third, various cognitive activities such as cultural values, attention, and anxiety all have a profound effect on the pain experience. These cognitive functions must be able to act selectively on the sensory and motivation systems in order to influence the pain response. The dorsal column-medial lemniscal system and dorsolateral projection pathways rapidly carry impulses directly and indirectly to the cerebral cortex. Influences that descend from the cortex are known to act at the level of the ventrobasal thalamus as well as dorsal horn cells. Therefore, these rapidly conducting ascending and descending systems appear to have the capacity to influence pain related information being transmitted over the more slowly conducting pathways and thus account for the fact that psychological processes play an important role in determining the quality and intensity of pain.

Melzack and Casey (1968) suggest that these three psychological processes interact with one another to provide perceptual information regarding the location, magnitude, and spatiotemporal properties of the noxious stimulus; motivational tendency toward escape; and cognitive information based upon past experience and probability of

outcome of the various response. All three components then influence the appropriate motor mechanisms which produce the overt behaviors characterizing the pain response.

#### 1.1.7 Importance of Pain

Pain is of crucial biological importance; true value of this can be seen in congenitally insensitive individuals who suffer excessive tissue damage such as burning a hand or arm on a heated stove, chewing off the end of their tongues, or chopping a knee with an axe all because of the inability to experience pain which would have protected them from these various occurrences (Swanson et al., 1965). Although pain does serve as a warning of injury to the individual and is of great diagnostic value to the physician it may also be an unnecessary evil at times when its warning effect is useless such as in terminal cancer or phantom limb pain.

#### 1.1.8 Treatment of Pain

##### 1.1.8.1 Pharmacological Intervention

The ideal analgesic agent should be effective when given orally, rapidly active after ingestion, and sufficiently strong to produce an appropriate level of analgesia; it should not produce tolerance, addiction, or respiratory depression; its actions should be specific and associated with few side effects; finally, an antidote should be available. In other words the ideal analgesic should control pain without producing any undesirable side effects and dependence (Villaverde and

MacMillan, 1977).

The narcotic analgesics are the group of drugs which, among analgesics available today, fulfill the above criteria most closely, even though they are far from being ideal; most of them are opium derivatives while others are synthetic or semisynthetic products; morphine and meperidine are the principal members of these two groups, respectively.

Besides producing analgesia the narcotics also produce, even when used in therapeutic doses, undesirable side effects such as sleepiness, nausea, vomiting, and respiratory depression. Among serious problems associated with the narcotics is that of the tolerance which develops to the analgesic effects upon repeated administrations; that is, continually increasing doses of narcotic must be administered to produce a sufficient analgesia. Unfortunately, in the chronic pain patient the doses of narcotic required are often extremely high increasing the frequency and severity of the associated side effects. In addition, as tolerance further develops there is no dose of narcotic which will produce analgesia short of causing death.

In addition to tolerance, physical dependence, i.e. addiction, develops with repeated use of narcotics; the abuse liability and possibility of developing psychological dependence on the effects of these compounds is one of the major limitations for their clinical use. However, in patients with painful terminal illnesses, this factor should not prevent the physician from alleviating the patient's pain and discomfort.

The principal use of the narcotics is for the treatment of severe pain which cannot be alleviated by other analgesics, such as in cancer, traumatic accidents, burns, fractures, and severe visceral lesions.

The salicylates, most notably acetylsalicylic acid (aspirin), constitute a second major group of analgesics. The overall analgesic effect of the salicylates is inferior to that achieved by the narcotics; salicylates do appear to be more effective against integumental than visceral pain.

While used in therapeutic doses, the salicylates do not produce respiratory depression or sleepiness, prolonged salicylate use may cause hyperventilation, tinnitus, and mental confusion. Gastrointestinal distress, nausea, and vomiting, are the most common side effects. Salicylates are recommended for treatment of headache, arthritis, dysmenorrhea, and neuralgia.

Para-aminophenol derivatives such as phenacetin and acetaminophen and pyrazolon derivatives such as antipyrine and aminopyrine and their modern congeners such as indomethacin all produce an equivalent level of analgesia as do the salicylates and consequently these compounds are employed in the treatment of similar types of pain as are the salicylate drugs. It should be emphasized that with many of these drugs their analgesic effect may depend on their peripheral antiinflammatory action. Thus, they may be useful in arthritis and gout.



In addition, high doses of phenacetin produce methemoglobinemia, nephropathy and hepatic necrosis while high doses of acetaminophen may cause thrombocytopenia and nephrotoxicity. However, in recommended therapeutic doses, phenacetin and acetaminophen are well tolerated.

Aminopyrine and possibly antipyrine produce severe blood dyscrasias such as thrombocytopenia and agranulocytosis; consequently, these compounds are reserved for use in cases that do not respond to salicylates or para-aminophenol derivatives.

Antimalarials, most notably quinine, whose main use is for the treatment of malaria also produce analgesia. As analgesics, however, their effects are weaker than those of the salicylates. Quinine is used only with considerable caution due to the potential nephrotoxic and hemolytic idiosyncratic reactions.

Finally, certain groups of drugs are used for specific types of pain as follows: (a) local anesthetics such as procaine, lidocaine, and dibucaine are used primarily to produce surface and infiltration anesthesia in localized parts of the body; (b) vasodilators such as nitroglycerine and amyl nitrate are used for the treatment of angina pectoris, both as a preventive and for relief of the acute attack; (c) corticoids such as cortisone, hydrocortisone, and prednisone are employed in the treatment of pain associated with inflammation, arthritis and bursitis; and (d) muscle relaxants such as meprobamate are used to treat pain associated with muscle spasms in rheumatic disease

or following trauma.

While numerous analgesic agents are employed for the relief of pain, the narcotic analgesic compounds constitute the most effective and most potent analgesics available today. In view, however, of their side actions and addictive liability, improvements in the pharmacological manipulation of pain, particularly chronic pain, are required.

#### 1.1.8.2 Neurosurgical Ablative Techniques

Various neurosurgical ablative procedures such as dorsal rhizotomy, chemical sympathectomy, percutaneous chordotomy, and chemical hypophysectomy have been employed, without a great deal of success, in the management of chronic pain.

Dorsal rhizotomy, sectioning of the dorsal roots, failed to produce long term (6 month) analgesia in the majority of cases (Loeser, 1974). Aside from imperfect surgical procedures, several other explanations may account for this lack of success. First, the source of pain may not lie in the periphery, therefore, deafferentation would not isolate the brain from the pathology (Melzack and Loeser, 1978). Second, deafferentation may itself generate abnormal firing patterns which the patient senses as painful (Loeser, 1974). Finally, primary afferent sensory nerve fibers associated with nociceptors are known to exist in the ventral roots (Applebaum et al., 1976); the presence of these fibers also may account for the failure of dorsal rhizotomy to relieve pain (Clifton et al., 1976). Overall the best prognosis

for the patient offered dorsal rhizotomy for the relief of chronic pain is poor.

Similarly, the localized injection of phenol (6.7% in water) into the sympathetic chain, chemical sympathectomy is useful only when the disease is confined within the visceral cavity; it therefore is rarely successful in pain of malignant disease in which the cells at any early stage invade neighboring structures innervated by the somatic nerves of the body wall (White, 1974). Furthermore, chemical sympathectomy suffers from a high incidence of associated complications such as pneumothorax, kidney puncture, and intravascular injections as well as a postoperative neuritis in the groin area and thigh regions of a large number of patients (14.6%; Reid et al., 1970).

Radiofrequency currents are used to produce lesions in the anterolateral quadrant of the spinal cord for the relief of chronic pain. Initially pain relief is excellent for 90% of the patients (n=1,279); however, an abrupt decrease in efficacy occurs three months after the operation and continues to decrease thereafter so that only 40% of the patients continue to report adequate pain relief one year after the operation (Rosonoff, 1974). In addition, complications such as muscle weakness, sphincter paralysis, and localized burning sensations have also been reported (Mullan et al., 1963). One explanation which may account for this procedural failure is that ascending tracts convey impulses arising from noxious stimuli are not restricted to the anterolateral quadrant of the spinal cord (see section 1.1.4.4).

Chemical hypophysectomy, neuroadenolysis, is achieved by the localized injection of absolute ethanol into the pituitary gland (Morrica, 1974). This procedure was initiated in cancer patients with diffuse bone and/or visceral metastasis arising from hormone-dependent tumors (Morrica, 1974). Miles and Lipton (1976) reported that cancer pain arising from other nonhormone-dependent tumors was also relieved by chemical hypophysectomy. However, since ethanol injected into the pituitary has been shown to spread to the hypothalamus, it may act there to interfere with pain perception (Miles and Lipton, 1976; Yanagida et al., 1979). Finally, the severe complications associated with chemical hypophysectomy such as diabetes insipidus, hypoadrenalism, hypothyroidism, decreased libido, hyperthermic crisis, hyperphagia, and anhidrosis suggest that this procedure be used only after other treatment procedures have failed to relieve pain (Morrica, 1976).

Overall, the neurosurgical ablative techniques have the disadvantages of being restricted to a specific part of the body, gradual return of pain with time, or more seriously, various permanent neurological complications. Thus, these methods for pain relief are gradually being replaced by other related methods namely nerve stimulation techniques.

#### 1.1.8.3 Stimulation Produced Analgesia (SPA)

Reynolds (1969) first employed electrical stimulation of the mid-brain periaqueductal grey matter (PAG) to render rats sufficiently antinociceptive to perform abdominal surgery. Since then, PAG stimu-

lation-induced analgesia (SPA) in laboratory animals has been reported to block behavioral responses such as extreme pinch (Reynolds, 1969), tissue damaging heat (Mayer and Liebeskind, 1974), electric tooth pulp stimulation (Oliveras et al., 1974), and the application of various algesic substances (Melzack and Melinkoff, 1974). Electrical stimulation of other central sites such as the septal nuclei, dorsolateral thalamic nuclei were effective with regard to only certain types of noxious stimuli (Mayer and Liebeskind, 1974); stimulation of the ventrobasal complex of the thalamus and lateral hypothalamus was completely ineffective (Mayer and Liebeskind, 1974).

In humans, electrical stimulation of various areas in the CNS such as the dorsal columns (Shealy et al., 1967), internal capsule (Adams et al., 1974), and PAG (Richardson and Akil, 1977a, b) produced analgesia. However, the occurrence of undesirable side effects such as nystagmus, nausea, vertigo, reports of enhanced pain sensations as well as the lack of prolonged effectiveness of treatment has limited the clinically effective target site to an area between the nucleus parafascicularis and third ventricle at the level of the posterior commissure (Richardson and Akil, 1977a, b).

SPA can be measured after only a few seconds of central stimulation (Mayer et al., 1971) and the post stimulation duration of effectiveness in the rat lasts for minutes to hours (Mayer and Liebeskind, 1974). Similarly, 30 minutes of stimulation afforded 3 to 4 hours of pain relief in humans (Hosobuchi et al., 1977). Thus, SPA provides an alternative approach for the control of chronic intractable pain.

Evidence suggests that SPA, morphine-induced antinociception, and the endogenous opioid system all may be interrelated. The mechanism involved in SPA as well as the role played by the endogenous opioid system in antinociception and analgesia will be reviewed subsequently (see sections 1.3.4 and 1.4.4).

#### 1.1.8.4 Psychological Manipulations

The first nonphysical method employed in the treatment of chronic pain utilizes placebos, pharmacologically inert agents. Placebo-induced analgesia reduced pain by about half of its original intensity in a variety of clinical situations for about 30% of the patients studied (Evans, 1974). On the other hand, placebos reduced experimentally-induced pain in only 3 to 16% of the experimental populations (Evans, 1974). Thus, placebos relieve pathological pain more effectively than they relieve experimental pain. It should be pointed out here that the placebo was at least half as effective as the assumed strength of the analgesic drug being administered under double blind conditions regardless of what analgesic was administered (Evans, 1974). This finding holds true for aspirin (Evans, 1974), morphine (Evans, 1974), and transcutaneous electrical stimulation (Thorsteinsson et al., 1978).

Overall, the judicious use of placebo-induced analgesia for a specific group of patients appears to be useful and in some cases may be more clinically effective than specific drugs. In addition, certain variables within the doctor patient relationship such as trust, belief, and the drug giving ritual appear to produce powerful curative

effects and therefore should be emphasized as a foundation on which all other therapeutic procedures concerning the treatment of chronic pain be built.

A second nonphysical method used for the treatment of chronic pain is called hypnosis-induced analgesia. The usefulness of hypnosis in the relief of pain was demonstrated early in the 19th century when limb amputations and other major operations were performed apparently painlessly with hypnosis as the only analgesic-anesthetic agent (Esdaile, 1957 cited by Hilgard, 1978). Since hypnotizability is seen as a personality trait which an individual possesses rather than a skill which may be improved with time (Perry, 1977), the range of patients in which hypnosis-induced analgesia would likely be effective is severely restricted.

Since naloxone fails to antagonize hypnosis-induced analgesia (Goldstein and Hilgard, 1975), it does not appear that the endogenous opioid system is involved in mediating this effect. Hilgard (1978) suggests that hypnosis-induced analgesia occurs primarily at higher cognitive levels which include cortical involvement.

The final nonphysical technique used in the treatment of chronic pain employs operant conditioning methods. Pain behavior, like other operants, while initially elicited by an antecedent stimulus may come under the control of consequences (Fordyce et al., 1973). When an operant is followed by a positive consequence such as praise, attention, money, or food that behavior is likely to occur in the future.

Alternatively, when an operant is followed by a negative consequence such as criticism or loss of valued rewards that behavior is likely to occur less in the future. Thus, in a specific group of patients, pain behavior may occur only because it is followed by positive consequences. Fordyce et al. (1973) successfully employed the operant conditioning technique in a study utilizing a selected group of patients who suffered from chronic pain which was thought to occur from factors other than tissue pathology. The results of this study demonstrated (i) a significant decrease in medication required and (ii) significant increases in walking and nonreclining activities.

## 1.2 Cholinergic System in Antinociception and Analgesia

### 1.2.1 Central Cholinergic System

Central actions of cholinergic agonists and antagonists, anticholinesterases, and acetylcholine (ACh) itself must be considered in terms of the presence and distribution of cholinergic synapses or cholinceptive neurons or both, as well as in terms of cholinergic pathways.

A variety of histochemical and biochemical techniques and binding studies have been utilized to investigate brain neuronal mechanisms in which ACh is thought to be the neurotransmitter. Acetylcholinesterase (AChE) has been investigated utilizing histochemical procedures (Shute and Lewis, 1967; Lewis and Shute, 1967) while ACh (Cheney et al., 1975), choline acetyltransferase (CAT; Kobayashi et



al., 1975), and high affinity choline uptake (Kuhar et al., 1973a, 1975) have been investigated by various biochemical tests. More recently, binding studies as well as studies of muscarinic and nicotinic cholinergic receptors have been used to determine the distribution of muscarinic (Kobayashi et al., 1978a; Krnjevic, 1974) and nicotinic (Hunt and Schmidt, 1978) binding sites. Since all of the aforementioned parameters are considered to be functional components of cholinergic neurons and synapses, indirect evidence from these studies concerning the distribution of cholinergic synapses and pathways can be obtained.

Shute and Lewis (1967) demonstrated that AChE accumulated at the proximal end of sectioned nerves whereas it disappeared distal to the division. Utilizing histochemical procedures, Shute and Lewis (1967) and Lewis and Shute (1967) mapped the cell bodies and axons of cholinergic neurons in the CNS. Two principal cholinergic pathways emerged from these studies. First, the ascending reticular system arises from reticular and tegmental nuclei of the brainstem and projects via dorsal and ventral tegmental pathways to virtually all cortical and subcortical structures. Second, the cholinergic limbic system consisted of septo-hippocampal connections as well as hippocampal afferent projections to the medial cortex, nuclei connecting with the ascending cholinergic reticular system, and subfornical and supraoptic crest.

Electrolytic lesions of the medial septal area decreased ACh levels, CAT activity, and high affinity choline uptake in the hippocampus (Kuhar et al., 1973a). Identical effects were observed in the

interpeduncular nucleus area when the medial habenular areas was lesioned (Kuhar et al., 1975). This provides additional evidence for cholinergic septo-hippocampal and habenular-interpeduncular tracts.

Utilizing mass fragmentography and gas chromatography, Cheney et al. (1975) demonstrated that a high ACh/CAT ratio which is indicative of cholinergic terminals was found in (i) olfactory structures, (ii) preoptic nuclei, (iii) several hypothalamic and thalamic nuclei, (iv) habenular and mammillary nuclei, (v) various midbrain nuclei, (vi) locus coeruleus, and (vii) nucleus interpositus of the cerebellum. In addition, high content of CAT has been demonstrated in certain cranial nerve nuclei including III, IV, VII, and XII as well as nuclei salivatorius, tractus solitarius and caudate putamen of the rat brain stem (Kobayashi et al., 1975).

Utilizing a binding assay, Kobayashi et al. (1978a) found a high density of cholinergic muscarinic binding sites in the hippocampus, caudate-putamen, nucleus accumbens and cerebral cortex; septal areas, interpeduncular nuclei, habenular nuclei, and medial thalamus exhibited a less dense population of binding sites; least dense binding site distribution was observed in the spinal cord and cerebellum.

The distribution of cholinergic nicotinic binding sites has also been investigated utilizing binding assays (Hunt and Schmidt, 1978). Binding sites were predominantly found with central areas of the brain associated with direct sensory input such as olfactory bulbs, superior colliculus, cochlear nuclei, the substantia gelatinosa, and the prin-

cipal trigeminal sensory nucleus (Hunt and Schmidt, 1978). Certain limbic areas such as the hippocampus, amygdala, mammillary bodies, and dorsal tegmental nucleus also contain nicotinic binding sites (Hunt and Schmidt, 1978).

Presynaptic localization of a neurotransmitter substance must be considered in terms of the concept of the synaptic vesicle. Accordingly, ACh has been shown to be contained in synaptic vesicles obtained from the synaptosomal fraction of cerebral cortical tissue (Whittaker and Sheridan, 1965) and of other brain tissues. Altogether, localization of ACh-containing vesicles agrees with that of other markers of the cholinergic system. It must be added in this context that ACh-containing vesicles are morphologically specific and differ from those containing GABA or catecholamine (Karczmar, 1976).

Localized injections of ACh and subsequent recordings from various cholinceptive neurons have provided additional information concerning the central cholinergic system. Most cholinceptive cells in the CNS were excited and depolarized; occasionally inhibitory, hyperpolarizing responses were observed (Krnjevic, 1974). The most common excitatory action of ACh in the CNS has muscarinic characteristics; it is relatively slow in onset and very prolonged and is readily blocked by atropine; the ionic mechanism of depolarization is probably a reduction in potassium conductance (Krnjevic, 1974). Other excitatory effects occur at nicotinic sites: ACh has a very quick and rapidly reversible effect on some CNS neurons such as the Renshaw cell; dihydro-beta-erthroidine and mecamlamine blocked this effect (Krnjevic, 1974).

Finally, inhibition of some CNS neurons caused by a specific increase in potassium permeability is mediated by muscarinic receptors (Krnjevic, 1974).

It should be pointed out that the presence of cholinceptive neurons, ACh and AChE by themselves do not insure proof of cholinergic transmission. For instance, the cerebellum contains AChE containing cells and fibers but no specific pathway in the cerebellum has been shown to act by release of ACh (Krnjevic, 1974).

Finally, ACh was the first neurotransmitter substance collected following stimulation of different CNS preparations (Pepeu, 1973). The original method of Mitchell (1963) for studying ACh release from the cerebral cortex utilized the cortical cup; ACh content was determined by bioassay on the dorsal muscle of the leech. Investigations of ACh release from subcortical structures were made possible by the push-pull cannula described by Gaddum (1961). In either case, ACh release studies were only possible if 95% of the AChE present had been previously inhibited (Lancaster, 1971). ACh release has been demonstrated in such brain areas as sensory motor, auditory, parietal, and visual cortex; caudate nucleus; ventrobasal complex of the thalamus; thalamic nuclei ventralis, lateralis, and posterior; hypothalamus; medulla; and spinal cord (Pepeu, 1973). In addition, ACh release from central brain sites depends upon the presence of calcium ions (Randic and Padjen, 1967). Overall, the release studies provide additional evidence and information concerning the distribution of the central cholinergic system.

In summary, what emerges is the concept that cholinergic synapses and pathways are present in many CNS structures; the major central cholinergic system comprises a diffuse ascending tegmental-mesencephalic-cortical system; the most notable pathways included here are the ventral and dorsal tegmental pathways, habenulo-interpeduncular tract, septohippocampal tract, and thalamocortical tract.

#### 1.2.2 Interactions with Other Neurotransmitter Systems

Next, the interaction between the cholinergic, catecholaminergic, and serotonergic neurotransmitter systems throughout the CNS has been demonstrated (Karczmar, 1975). Cholinergic agonists and antagonists affect levels and/or turnover rates of brain catecholamines and serotonin (5-HT); the effect on the 5-HT system is most pronounced (Karczmar, 1976). Diisopropyl phosphofluoridate (DFP) increased 5-HT levels in the rabbit midbrain, thalamus, hippocampus, and medulla (Barnes et al., 1974); effects were reversed by atropine (Barnes et al., 1975). DFP recently has been shown to increase both 5-HT levels and turnover in several rat brain regions; effects were reversed by atropine (Barnes et al., 1978).

Furthermore, DFP increased levels of dopamine (DA) in several rabbit brain parts including the thalamus, hypothalamus, midbrain, and hippocampus (Glisson et al., 1972, 1974). Alternatively, DFP decreased norepinephrine (NE) levels in the same four rabbit brain regions discussed above (Glisson et al., 1972, 1974). The effect of DFP on NE arises from central actions while the DFP effect on DA depends in part

on peripheral actions; atropine methyl nitrate, the quaternary analogue which does not readily penetrate the blood brain barrier (BBB) blocked the DFP effect on DA but was ineffective against NE (Glisson et al., 1974).

Concerning reciprocal relationships, ACh-5-HT interactions may be bidirectional. 5-hydroxytryptophan (5-HTP), the metabolic precursor for 5-HT, significantly increased the level of ACh following pretreatment with the serotonergic neurotoxin 5,6-dihydroxytryptamine in mice (Barnes et al., 1973a, b). On the other hand, ACh brain levels were not affected by the catecholamine neurotoxin, 6-hydroxydopamine or by the metabolic precursor for catecholamines, 1-dopa (Barnes et al., 1973c).

The pharmacological evidence described above demonstrates that the interaction between various neurotransmitter systems in the CNS is widespread. The exact mechanism of these effects, in terms of neurotransmitter turnover rates and pertinent circuitry remains to be established. In addition, the results discussed above suggest that it may be difficult to obtain a predictable cholinergic effect that would depend solely on cholinergic synapses and pathways and/or cholinceptive neurons. However, specific effects of cholinergic drugs on behavior are obtained as discussed in the following sections.

### 1.2.3 Antinociception and Analgesia

#### 1.2.3.1 Compounds Which Affect the Cholinergic System

Several cholinomimetic compounds such as dibromopyruvic acid (Martin et al., 1958), tremorine (Chen, 1958), arecoline (Herz, 1961), oxotremorine (Harris et al., 1968), carbachol (Metys et al., 1969), and pilocarpine (Houser and Van Hart, 1973); various anti-cholinesterase agents such as physostigmine (Harris et al., 1968) and DFP (Koehn and Karczmar, 1978); and ACh itself (Pedigo et al., 1975) produce antinociception when administered to mice and rats.

Recently, physostigmine (Sitaram et al., 1977) and arecoline (Sitaram, 1979, personal communication) have caused an analgetic action in normal human volunteers.

Scopolamine, an anticholinergic agent, produced antinociception when administered to monkeys (Pert, 1975). The discrepancy between findings in the monkey and all other species including man concerning the involvement of the cholinergic system in nociception cannot be explained at present.

The antinociceptive and analgesic states produced by the compounds mentioned above will henceforth be referred to as cholinergic-induced antinociception and analgesia, respectively.

Prostigmine (Slaughter and Munsell, 1940), pilocarpine (Saxena and Gupta, 1958), DFP (Bhargava and Way, 1972), and ACh (Pedigo et al., 1975) potentiate narcotic-induced antinociception in laboratory animals. In addition, some partial agonists of narcotics such as cyclazocine, cyclorphan, nalorphan, and pentazocine are converted from inactive to active antinociceptive agents in the mouse in the presence of physostigmine (Harris et al., 1967).

Some of these various agents which affect the central cholinergic system proved to be more potent on a milligram basis than morphine; for instance, oxotremorine was 3000 times more potent than morphine in the mouse (Leslie, 1969). Finally, oxotremorine but not morphine could produce antinociception when administered to frogs (Nistri et al., 1974).

Several anticholinesterase agents such as pyridostigmine (Slaughter, 1950) physostigmine (Floodmark and Wrammer, 1945) and neostigmine (Hand and Audin, 1944; Christensen and Gross, 1948) potentiate narcotic-induced analgesia in humans. In fact, the combination of neostigmine with a narcotic analgesic was considered to be more effective than the narcotic analgesic used alone as the dose of the narcotic employed to produce analgesia could be reduced, hence lessening the severity of associated side effects, most notably nausea and somnolence (Hand and Audin, 1944).

#### 1.2.3.2 Central Muscarinic Nature

Cholinergic-induced antinociception and analgesia result from



actions involving central cholinceptive sites of the muscarinic type as suggested by the following. First, tertiary anticholinergic-antimuscarinic agents such as atropine sulfate and scopolamine which cross the BBB antagonize cholinergic-induced antinociception and analgesia; quaternary anticholinergics such as atropine methyl nitrate and methylscopolamine which do not cross the BBB are ineffective (Herz, 1961; Handley and Spencer, 1969; Pedigo et al., 1975; Sitaram et al., 1977; Koehn and Karczmar, 1978). On the other hand, various anticholinergic drugs which block central cholinceptive sites of the nicotinic type such as dihydro- $\beta$ -erythroidine (Herz, 1961), various alpha substituted acetylcholine derivatives (Dewey et al., 1975), nicotine and hexamethonium (Pert, 1975), and mecamylamine (Pedigo et al., 1975) do not affect cholinergic-induced antinociception. It may be added that in monkeys, only those anticholinergic agents such as scopolamine which cross the BBB are effective antinociceptive agents when administered systemically; methylscopolamine was ineffective (Pert, 1975).

Second, muscarinics, but generally not nicotinics (see however, below), do exert antinociception. For instance, carbachol, a quaternary cholinomimetic which also possesses some activity at nicotinic receptors, produces antinociception when administered intracerebroventricularly (Icvt); effects were reversed by atropine (Metys et al., 1969).

Drug interactions with muscarinic receptors in peripheral tissues appear to be stereospecific; (+) isomers of beta-substituted methylcholine mimic the action of muscarine; (-) isomers were inactive

(Ellenbroek and Van Rossum, 1960). Chemical derivatives of beta-substituted methylcholine which exhibit muscarinic action stereospecifically antagonized ACh-induced antinociception in mice; (+) isomers were active while the (-) isomers were inactive (Dewey et al., 1975). Thus, cholinergic-induced antinociception appears to be stereospecifically mediated by muscarinic receptors in the CNS.

It must be added that while most laboratories have failed to demonstrate antinociceptive activity for compounds which interact with nicotinic cholinergic receptors (Herz, 1961; Metys et al., 1969; Pert, 1975; Pedigo et al., 1975), Phan et al. (1973) showed that nicotine produced antinociception when administered to rodents; the effect was blocked by mecamylamine but atropine was ineffective. However, more research concerning this effect is required before two independent cholinergic systems can be implicated in the production of antinociception and analgesia.

Pharmacological evidence concerning specific central sites for cholinergic modulation of the antinociceptive state comes from many sources. Intraventricular (Ivt) administration of carbachol (Metys et al., 1969), oxotremorine (Handley and Spencer, 1969), ACh (Pedigo et al., 1975), and scopolamine (Pert and Maxey, 1975) suggest the involvement of structures immediately around the ventricular system since the time course of the antinociceptive state and diffusion properties of these drugs limit their potential sites of action to those particular areas. In addition, Ivt administrations of oxotremorine and arecoline into the septal area, mesencephalic reticular formation,

hypothalamus, and medial thalamic nuclei produce significant antinociception; applications into the striate or hippocampus were ineffective; effects were reversed by atropine (Metys et al., 1969).

The close anatomical proximity of the ascending cholinergic reticular formation and the paleospinothalamic and spinoreticular tracts which carry nociceptive information (see section 1.1.4.4), as well as the fact that Ivt applications of cholinomimetic agents into these areas produced antinociception led Sitaram et al. (1977) to postulate that physostigmine produced analgesia by virtue of its action mediated by the ascending reticular activating system.

In any case, the specific sites and pathways involved in mediating and modifying cholinergic-induced antinociception and analgesia remain to be further identified.

#### 1.2.3.3 Mechanisms

The mechanisms of effects concerning cholinergic-induced antinociception are not known. The following evidence suggests as well as refutes various possible explanations.

First, the production of antinociception and elevation of ACh levels may be correlated for some centrally acting cholinomimetics such as oxotremorine (Harris et al., 1969). Morphine which produces a potent level of antinociception also increases brain ACh levels (Hano et al., 1964). However, various partial narcotic agonists and antagonists such as nalorphine, naloxone, pentazocine, and cyclazocine

(Howes et al., 1969) as well as several CNS depressants such as methylparafynol, hydroxydione, pentobarbital, and reserpine (Giarmann and Pepeu, 1962) do not produce antinociception but increase brain ACh levels. Therefore, no correlation exists between the ability of a compound to produce antinociception and increase brain ACh levels. Thus, cholinergic-induced antinociception needs not be directly attributable to enhancement of brain ACh levels.

Alternatively, oxotremorine and morphine decrease the turnover rate of brain ACh (Norberg and Sundwall, 1977); this action of morphine appears to be due to its antirelease effect (Domino et al., 1976). However, pentobarbital also decreases brain ACh turnover (Norberg and Sundwall, 1977). Again, no correlation exists between the ability of a compound to produce antinociception and decrease ACh turnover.

It should be pointed out that correlations between the antinociceptive state and whole brain or brain region neurochemistry may be misleading. It may be that the pertinent neurochemical changes associated with antinociception are restricted to nondetectable brain sites. Alternatively, the compounds which affect the cholinergic system may interact with other neurotransmitter systems to produce antinociception. Since the various agents which affect the central cholinergic system influence the levels and turnover rates of other neurotransmitters in the CNS, nociception and pain are probably mediated by a complex interplay of neurotransmitters (see section 1.2.2). To

identify the neurotransmitter or neurotransmitters involved, various pharmacological manipulations were attempted as follows.

Pretreatment with reserpine which decreases brain DA, NE and 5-HT levels, antagonized the antinociceptive states produced by physostigmine (Pleuvry and Tobias, 1971) and tremorine (Sethy et al., 1971). Since reserpine decreases both catecholamines and indoleamines (Shore and Brodie, 1957), the following procedures were utilized to differentiate the involvement of the various neurotransmitters.

Concerning the involvement of DA and NE, diethyldithiocarbamate, which reduces brain NE levels by inhibiting dopamine-beta-hydroxylase (DBH), antagonized the antinociceptive state produced by tremorine (Sethy et al., 1971), physostigmine and oxotremorine (Pleuvry and Tobias, 1971), while alpha-methylparatyrosine (AMPT), a tyrosine hydroxylase inhibitor, 1-dopa, the metabolic precursor for DA and NE, and pimozide, a DA receptor blocker, were ineffective (Paalzow and Paalzow, 1975). These data suggest that NE may play a role in cholinergic-induced antinociception but that DA probably does not.

Various manipulations which affect the serotonergic system exert, on the whole, inconsistent effects on cholinergic antinociception. For instance, precursor loading with 5-HTP enhances physostigmine-induced antinociception in mice (Pleuvry and Tobias, 1971) but not in rats (Paalzow and Paalzow, 1975). Para-chlorophenylalanine (PCPA) which decreases brain 5-HT levels by inhibiting tryptophan hydroxylation, antagonizes physostigmine-induced antinociception in mice

(Pleuvry and Tobias, 1971) and rats (Bhattacharya and Nayak, 1978); however, PCPA had no effect on oxotremorine (Pleuvry and Tobias, 1971; Paalzow and Paalzow, 1975) or DFP-induced antinociception (Koehn and Karczmar, 1978) in rats. Overall, reports from various authors concerning the involvement of the serotonergic system in cholinergic-induced antinociception are contradictory and inconclusive. The involvement of the serotonergic system in antinociception and analgesia will be discussed in more detail in section 1.3.

Endogenous opioid system may constitute another system that could be involved in cholinergic-induced antinociception (see also section 1.4). Naloxone, a pure narcotic antagonist, antagonized the antinociceptive state produced by physostigmine and oxotremorine (Harris et al., 1969), ACh (Pedigo et al., 1975) and DFP (Koehn and Karczmar, 1978). Furthermore, antagonism of DFP-induced antinociception by stereoisomers of some narcotic antagonists was shown to be stereospecific (Koehn et al., 1979). On the other hand, Pedigo et al. (1975) demonstrated that inactive (+) isomers of partial narcotic agonists antagonized ACh-induced antinociception; (-) active isomers were ineffective.

Since one of the major characteristics of morphine-induced antinociception is the tolerance which arises on chronic treatment, studies utilizing a cross tolerance design were performed to discern a possible common mechanism of cholinergic- and morphine-induced antinociception. Invariably, cross tolerance did not develop between mor-

phine-induced antinociception and antinociceptive state produced by oxotremorine (Howes et al., 1969), physostigmine (Pleuvry and Tobias, 1971), scopolamine (Pert and Maxey, 1975), or DFP (Koehn et al., 1979). The results do not nullify the idea that morphine and the various compounds which affect the cholinergic system may be acting on opiate sensitive systems to produce their antinociceptive states since tolerance may not develop at sites at which the cholinomimetics and anticholinesterases act to produce antinociception. Overall, the data suggest the possible involvement of an endogenous opioid system in cholinergic-induced antinociception. The involvement of the endogenous opioid system in antinociception and analgesia will be discussed in greater detail in section 1.4.

In summary, the exact neurochemical mechanisms involved in mediating or modifying cholinergic-induced antinociception remain only poorly understood at this time.

#### 1.2.4 Locomotion and Exploration

Cholinergic agonists and antagonists induce or affect a number of animal locomotor and related overt behaviors including a state referred to as "alert non-mobile behavior" (ANMB; Karczmar, 1977).

Concerning the spontaneous locomotor activity in rats, ACh (Herman et al., 1972), arecoline (Costall et al., 1972), and physostigmine (Adams, 1973) decrease spontaneous locomotor activity; this effect was reversed by atropine. Alternatively, atropine sulfate and scopolamine increase spontaneous locomotion; the corre-

sponding quaternary compounds were ineffective (Adams, 1973). Thus, stimulation of central cholinergic muscarinic receptors attenuate while blocking those receptors enhances locomotion.

The locomotor depression produced by cholinomimetics and anti-cholinesterases appears to be primarily related to the relative activity of the dopaminergic and cholinergic systems in the nigrostriatal and extrapyramidal pathways; locomotion is mediated by cholinergic projections to the striatum which are behaviorally inhibitory; this system may be modulated via dopaminergic inhibition (Karczmar, 1977). However, locomotion may also depend upon a cholinergic inhibitory mechanism in the septo-hippocampal system. Atropine or scopolamine applied to dorsal or ventral hippocampal sites increased locomotor activity in the rat (Leaton and Rech, 1972) and mouse (Abeelen et al., 1972).

ANMB combines with what appears to be mental alertness; again, hippocampus may be the site involved as ANMB correlates with theta waves or hippocampal slow activity electroencephalographic (EEG) patterns (Karczmar, 1977). However, hippocampal slow theta wave may not be always concomitant with immobility or ANMB. In rats, the presence of hippocampal theta wave activity was correlated with attentional or investigational responses occurring during exposure to a novel environment (exploratory activity; Komisaruk, 1970). In addition, intra-hippocampal administrations of methylscopolamine increased exploratory activity, in two strains of mice; neostigmine was ineffective (Abeelen et al., 1972). Thus, exploration appears to be blocked by a cholin-



ergic inhibitory mechanism residing probably in septal-hippocampal pathways.

#### 1.2.5 Thermoregulation

Meyers (1974) described a scheme for the role of the cholinergic system in thermoregulation of that cat and monkey. According to this scheme, the anterior hypothalamic preoptic area contains neurons which are thermosensitive as well as a comparator mechanism which contrasts the set point with local temperature; 5-HT and NE are released within this area to activate and inhibit, respectively, a cholinergic heat production system which then passes through the posterior hypothalamus. The posterior hypothalamus contains a set point mechanism which depends upon the  $\text{Na}^+/\text{Ca}^{++}$  ratio, an aberration of which will activate an independent cholinergic heat dissipating system.

Evidence from pharmacological studies support this scheme. First, intracerebral administrations of carbachol (Avery, 1970) or ACh (Rudy and Wolf, 1972) into anterior hypothalamic sites produced hyperthermia. Alternatively, pilocarpine or ACh produced hypothermia when administered into posterior hypothalamic sites; effects were reversed by atropine (Kirkpatrick and Lomax, 1970). Cholinomimetics and anticholinesterases administered systemically produce hypothermia in the rat (Lomax and Jenden, 1966; Kirkpatrick and Lomax, 1970; Meeter and Wolthius, 1968); however, whether sites and/or mechanisms other than the hypothalamic thermoregulatory center are involved is undetermined at this time.

### 1.3 Serotonergic System in Antinociception and Analgesia

#### 1.3.1 Central Serotonergic System

Just as the central actions of drugs which affect the cholinergic system must be considered in terms of the associated central cholinergic structures, so too must drugs which affect the central serotonergic system be viewed in terms of corresponding serotonin (5-HT) receptors, synapses, and pathways.

Unlike the cholinergic cell bodies which exist in a very diffuse pattern throughout the CNS, cell bodies of 5-HT neurons are mainly localized in midline raphe nuclei of the lower brainstem. Utilizing fluorescent histochemical techniques, Dahlstrom and Fuxe (1964) identified nine distinct brainstem regions associated with 5-HT neurons; these regions were consecutively labelled B1 through B9 from the caudal medulla to the rostral pons.

B1, B2, and B3, namely raphe nuclei pallidus, obscurus, and magnus (NRM), respectively, are located in the ventromedial medulla and send axons down the spinal cord via anterior and lateral funiculi to terminate with 5-HT containing terminals in the spinal dorsal, ventral grey matter, and sympathetic chain (Dahlstrom and Fuxe, 1965; Basbaum et al., 1978). These nuclei and associated projection pathways constitute the descending 5-HT system.

5-HT neurons located in the central dorsomedial midbrain, namely raphe nuclei dorsalis (B7) and medianus (B8) project to the telenceph-

alon and diencephalon. The greatest number of ascending 5-HT fibers originate in the dorsal raphe (B7); striatal and hippocampal 5-HT projections arise chiefly in the dorsal and median raphe nuclei, respectively (Dahlstrom and Fuxe, 1964; Lorens and Guldberg, 1974). 5-HT cells in the region of the formatio reticularis (B9) located just ventral to B7 and B8 in the midbrain also project rostrally to the diencephalon (Dahlstrom and Fuxe, 1964).

Two groups of 5-HT cells are found in the pons; the raphe nucleus pontis (B5) lies at the level of the nucleus motorius and the B6 cell group is found on the midline just below the fourth ventricle; both B5 and B6 send fibers to the hypothalamus (Dahlstrom and Fuxe, 1964; Fuxe and Jonsson, 1974). The final group of 5-HT cells is located in the vicinity of the area postrema in the medulla; projection systems from this group remain to be identified (Dahlstrom and Fuxe, 1964). Altogether, the B4 through B9 cell groups and projection systems comprise the ascending 5-HT system.

The metabolic pathways involved in the synthesis and catabolism of brain 5-HT are outlined briefly as follows (Messing and Lytle, 1977). First, the amino acid, tryptophan, is taken up into the neuron and 5-hydroxylated by tryptophan hydroxylase (TH) to form the intermediate metabolite, 1-5-hydroxytryptophan (5-HTP); the intracerebral hydroxylation of tryptophan is the primary factor and rate limiting reaction controlling cerebral 5-HT formation (Moir and Eccleston, 1968). 5-HTP is then decarboxylated by the enzyme aromatic l-amino acid decarboxyl-

ase to form 5-hydroxytryptamine (5-HT; serotonin). Following release from the neuron, 5-HT is actively taken up by presynaptic nerve terminals where it is deaminated and oxidized by the enzymes monoamine oxidase and aldehyde dehydrogenase, respectively, to form the major metabolite, 5-hydroxyindoleacetic acid (5-HIAA).

### 1.3.2 Role of 5-HT in Antinociception and Analgesia

Various experimental procedures have been utilized to investigate a possible role of 5-HT in the production of antinociception and analgesia; included here are pharmacological studies, dietary manipulations, and electrical lesions experiments; electrical stimulation studies will be discussed in section 1.3.4.

Para-chlorophenylalanine (PCPA) decreases 5-HT and 5-HIAA levels by inhibiting TH, the rate limiting enzyme in 5-HT synthesis (Koe and Weissman, 1966). PCPA decreased jump response thresholds to electric shocks in rats; this effect was reversed by the administration of the metabolic precursor, 5-HTP (Tenen, 1967). 5-HTP produced antinociception when administered by itself to rats (Contreras and Tamayo, 1967; Radouco-Thomas et al., 1967). However, administration of 5-HTP increases 5-HT formation within 5-HT neurons but also leads to the formation of 5-HT within dopamine neurons (Fuxe et al., 1971). In contrast, l-tryptophan administrations increase 5-HT formation only in 5-HT neurons due to the relatively specific localization of the enzyme tryptophan hydroxylase (Moir and Eccleston, 1968). L-tryptophan administrations do not affect sensitivity to electric shocks in

rats (Hole and Marsden, 1975). Fluoxetine hydrochloride (Lilly 110140), a specific inhibitor of 5-HT uptake into synaptosome (Wong et al., 1974), increased jump response thresholds in rats (Messing et al., 1975). Quipazine, which mimicks the effects of 5-HT on various smooth muscle preparations (Hong et al., 1969), also produced antinociception when administered to rats; effects were antagonized by methysergide (Samanin et al., 1976). The major factor which prevents researchers from determining whether 5-HT participates in antinociception and analgesia is the lack of a specific 5-HT receptor blocker. Five drugs (cinanserin, cyproheptadine, methysergide, methergoline, and methiothepin) have been suggested to block 5-HT receptors in the brain based on their ability to block the effects of 5-HT on smooth muscle and invertebrate neurons; unfortunately, their 5-HT antagonism is not entirely specific (Haigler and Aghajanian, 1977).

Rats fed a tryptophan deficient diet exhibited low tryptophan and 5-HT levels and decreased flinch-jump thresholds; PCPA augmented the hypernociceptive state; administrations of tryptophan or fluoxetine restored flinch-jump thresholds to control levels (Lytle et al., 1975; Messing et al., 1976).

Electrolytic lesions in the median forebrain bundle, septum, ventrolateral tegmentum, and dorsomedial tegmentum decreased telencephalic 5-HT content and increased sensitivity to electric foot-shock in rats (Lints and Harvey, 1969; Harvey and Lints, 1971); these two

effects were reversed by the administration of 5-HTP; administrations of l-dopa were ineffective (Harvey et al., 1975).

While electrolytic lesions of specific CNS structures such as the median forebrain bundle decrease brain 5-HT content and produce hypernociception, the electrolytic lesioning process itself probably destroys non-serotonergic neurons as well. In addition, when 5-HT neurons in the dorsal (B7) or median raphe (B8) are destroyed by electrolytic lesions no alteration in nociceptive responses in rats were observed (Harvey et al., 1974; Lorens and Yunger, 1974).

In summary, while some evidence suggests that 5-HT participates in antinociception and analgesia, no conclusive evidence for the relationship exists.

### 1.3.3 Serotonin (5-HT) and Morphine-Induced Antinociception

Many of the various pharmacological and physiological manipulations which are known to affect the central serotonergic system and the nociceptive state also affect morphine-induced antinociception.

Several compounds which affect the serotonergic system have been shown by a variety of testing procedures to potentiate narcotic-induced antinociception; included here are 5-HTP (Contreras and Tamayo, 1967; Radouco-Thomas et al., 1967), fluoxetine (Messing et al., 1975), as well as 5-HT itself administered intraventricularly (Ivt; Sewell and Spencer, 1974). Pretreatment with PCPA antagonized morphine-induced antinociception; this effect was reversed by 5-HTP (Gorlitz

and Frey, 1972). Methysergide, cinanserin, and cyproheptadine also antagonized morphine-induced antinociception (Gorlitz and Frey, 1972; Yaksh et al., 1976a).

Electrolytic lesion studies have produced varying effects on morphine-induced antinociception depending upon the lesion placement. Lesions that destroy specific ascending serotonergic fiber systems, as demonstrated by the resultant decrease in telencephalic 5-HT content, fail to alter the antinociceptive action of morphine in the rat: sites included here are (i) nucleus raphe medianus (B8; Lorens and Yunger, 1974; Hole and Marsden, 1975), nucleus raphe dorsalis (B7; Lorens and Yunger, 1974; Hole and Marsden, 1975), dorsal and ventral tegmental nuclei of Gudden (Lorens et al., 1975), and median fore-brain bundle (Harvey et al., 1975).

On the other hand, electrolytic lesions of the nucleus raphe magnus (B3) antagonized morphine-induced antinociception in the rat (Proudfit and Anderson, 1975). Since the nucleus raphe magnus (B3) is known to contain 5-HT neurons which descend to terminate on spinal cord structures known to respond to noxious stimuli (Proudfit and Anderson, 1975; Basbaum et al., 1978), the descending serotonergic system may participate in the expression of morphine-induced antinociception.

#### 1.3.4 Serotonin(5-HT) and Stimulation Produced Analgesia (SPA)

Drugs which affect the serotonergic system have been shown to alter stimulation produced analgesia (SPA) similarly to their effect

on morphine-induced antinociception (see section 1.3.3). Thus, PCPA antagonized the antinociceptive state produced by morphine (Gorlitz and Frey, 1972) as well as by electrical stimulation (SPA) of the nucleus raphe dorsalis (B7) in the rat (Akil and Mayer, 1972).

Similarly, electrical stimulation of the nucleus raphe magnus (B3) also produced antinociception in the rat (Proudfit and Anderson, 1975). Related studies have shown that selective lesions of the spinal cord dorsolateral funiculus (DLF) prevents the antinociceptive actions of SPA applied to periaqueductal grey sites (PAG) or of morphine (Basbaum et al., 1977). Similarly, DLF lesions prevent the inhibitory effect of nucleus raphe magnus (B3) on spinal cord neurons in laminae I, II, and V which are considered to be involved in pain modulation (Fields et al., 1976; see section 1.1.4.3). Recently, serotonin-containing fibers which descend from the nucleus raphe magnus (B3) and terminate in lamina I, II, IV, and V have been shown to descend through the DLF (Basbaum et al., 1978). These particular studies along with those described in section 1.4.4 illustrate the importance of the descending serotonergic system underlying the antinociceptive actions of SPA and morphine administrations.

While the serotonergic system appears to participate, at least in part, in mediating SPA and morphine-induced antinociception, it is also apparent that other neurotransmitter systems also mediate these effects. Pharmacological manipulations of catecholamine systems alter SPA; pimozide, a dopamine receptor blocker, decreased while apomorphine, a dopamine receptor stimulator increased SPA; disulfiram,



which depletes NE by inhibiting the enzyme dopamine-beta-hydroxylase (DBH), increased SPA (Akil and Liebeskind, 1975). NE specifically reduced the firing rate of dorsal horn interneurons in response to noxious stimuli but did not alter the firing rate in cells excited by innocuous stimuli (Belcher et al., 1978). Recently, Yaksh (1979) reported that the antinociceptive action of morphine applied to PAG sites could be antagonized only by the combined intrathecal administrations of phentolamine, a NE receptor blocker, and methysergide; administrations of either antagonist alone significantly although incompletely antagonized this morphine effect. Clearly, non-serotonergic systems exist which participate in the expression of SPA and morphine-induced antinociception.

#### 1.4 Endogenous Opioid System in Antinociception and Analgesia

##### 1.4.1 Endogenous Opioid System

One of the more interesting findings in neuroscience research has been the discovery of stereospecific opiate binding sites in the CNS (Goldstein et al., 1971) and subsequent isolation and identification of several endogenous opioid peptides (Hughes et al., 1975a; Guillemin et al., 1976; Li and Chung, 1976). The endogenous opioid peptides subsequently have demonstrated opiate-like activities, particularly the ability to produce antinociception, when administered to laboratory animals (see section 1.4.2).

The first endogenous opioids found in the brain and sequenced by Hughes et al. (1975a) were the pentapeptides, methionine- and leucine-

enkephalin, which differed from each other only in the terminal amino acid. Subsequently, other larger peptides found in the brain and pituitary exhibited similar opiate action; included here are beta ( $\beta$ )-endorphin (Li and Chung, 1976) and alpha ( $\alpha$ )- and gamma ( $\gamma$ )-endorphin (Guillemin et al., 1976).

The endorphins each share a common amino acid sequence with the terminal residues of  $\beta$ -lipotropin (LPH), a 91 amino acid pituitary peptide discovered by Li et al. (1965); residues 61 to 91, 61 to 76, and 61 to 77 of  $\beta$ -LPH are identical with  $\beta$ -,  $\alpha$ -, and  $\gamma$ -endorphin, respectively. Finally, the structure of methionine-enkephalin occupies position 61 to 65 of B-LPH.

Goldstein (1976) suggested that  $\beta$ -LPH might be enzymatically cleaved in the pituitary to form  $\alpha$ -,  $\beta$ -, and  $\gamma$ -endorphin and, in turn, methionine-enkephalin. However, it has recently been shown that hypophysectomy failed to alter the quantity and distribution of the endorphins (Cheung and Goldstein, 1976) or enkephalins (Kobayashi et al., 1978b) in the brain suggesting that the pituitary and brain constitute separate compartments with respect to these peptides. Presently, research aimed at identifying possible prohormones and/or precursors for the various opioid peptides is being conducted (Yang et al., 1979).

The enkephalin content of various CNS structures has been shown to be unevenly distributed by a variety of assay procedures including bioassay (Hughes et al., 1977), radioimmunoassay (Yang et al., 1977;

Kobayashi et al., 1978b), and receptor binding studies (Simantov et al., 1976a). In general, the striatum, central grey region, nucleus accumbens, and hypothalamus contained the highest content of methionine- and leucine-enkephalin; the thalamus, amygdala, pons, medulla, and caudate-putamen contained lesser amounts of the enkephalins; the midbrain, hippocampus, cerebellum, and cortex exhibited the lowest enkephalin content (Hughes et al., 1977; Yang et al., 1977; Kobayashi et al., 1978b; Simantov et al., 1976a). In every brain region studied, the methionine-enkephalin content was found to be 2 to 7 times greater than the corresponding leucine-enkephalin content (Kobayashi et al., 1978b). In addition, immunohistochemical studies have also demonstrated the presence of methionine- and leucine-enkephalin positive fibers in laminae I, II, III, V, and VII of the spinal cord (Watson et al., 1977; Simantov et al., 1977).

On the other hand,  $\beta$ -endorphin-like immunoreactivity is seen predominantly in the hypothalamus, septum, midbrain, and pons-medulla while no material attributable to  $\beta$ -endorphin could be found in the striatum, hippocampus, cortex, or cerebellum (Rossier et al., 1977b; Watson et al., 1978). Overall, the distribution of a  $\beta$ -endorphin reactive system and a methionine-enkephalin reactive system in the CNS appear to be quite different suggesting the existence of two separate opioid peptide systems.

Opiate binding sites and opioid peptide containing cell bodies and/or terminals have been found for the most part to be distributed

in close proximity to each other. For example, in accordance with the relative distributions of opioid peptides, the periaqueductal grey region, several hypothalamic nuclei, and spinal cord laminae I, II, and III exhibited high density of opiate binding sites; the cortex and cerebellum contained the least dense opiate binding sites (Kuhar et al., 1973b; Atweh and Kuhar, 1977a, b). On the other hand, the nucleus raphe magnus (NRM) contains a high level of methionine-enkephalin positive cells (Hokfelt et al., 1977) but exhibits a low level of opiate binding sites (Atweh and Kuhar, 1977a).

Subcellular fractionation experiments demonstrated that enkephalin-like-activity was predominantly localized to the synaptosomal fraction, the region known to contain several neurotransmitters (Simantov et al., 1976b).

Finally, methionine- and leucine-enkephalin are released from synaptosomes and isolated striatal slices by potassium-induced neuron depolarization or veratridine (Henderson et al., 1978);  $\beta$ -endorphin is released from pituitary as well as hypothalamic sites by potassium-evoked cellular depolarization (Przewlocki et al., 1978; Osborne et al., 1979).

Overall, the enkephalins and endorphins fulfill two criteria required for identity as a neurotransmitter substance. First, these peptides exhibit the differential distribution expected of a neuronal system in the CNS. Second, pharmacological antagonism establishes whether the neurotransmitter is released and characterizes the

receptor mediating the various responses (see sections 1.4.2 and 1.4.3). However, several other criteria such as physiologically-induced release, vesicular location, transmitter synthesizing system, and identity of action must be met before their role as neurotransmitter substances is established.

#### 1.4.2 Antinociception, Analgesia, Enkephalins, and Endorphins

The enkephalins and  $\beta$ -endorphin have been shown to produce a dose dependent, naloxone reversible, antinociceptive state following intracerebroventricular (Icvt) administrations in the rat (Belluzzi et al., 1976; Bloom et al., 1976), mouse (Buscher et al., 1976; Loh et al., 1976), and cat (Meglio et al., 1977).

Enkephalin-induced antinociception develops after a 2 to 3 minute latency and lasts for 5 to 10 minutes (Buscher et al., 1976); antinociception produced by  $\beta$ -endorphin lasts up to 90 minutes after administration (Loh et al., 1976). It appears that the short duration of enkephalin-induced antinociception and, in fact, the failure of some investigators to demonstrate any antinociceptive action (Bloom et al., 1976) is attributable to the fact that the enkephalins, unlike  $\beta$ -endorphin, are rapidly destroyed enzymatically in the brain (Dupont et al., 1977). Structurally altered enkephalins, most notably D-al<sup>2</sup>-methionine-enkephalin, which are resistant to enzymatic degradation are active antinociceptive agents when administered systemically or orally (Roemer et al., 1977).

The enkephalins and  $\beta$ -endorphin also produce antinociception in the rat when administered directly into the midbrain periaqueductal grey region (Chang and Fong, 1976; Malick and Goldstein, 1977) or intrathecally (Yaksh and Henry, 1978; Yaksh et al., 1978).

Finally, methionine-enkephalin reduced the firing rate of dorsal horn neurons activated by noxious stimuli but did not affect the firing rate of neurons activated by innocuous stimuli; inhibitory effects were reversed by naloxone (Randic and Miletic, 1978).

#### 1.4.3 Antinociception, Analgesia, and Naloxone

Jacob et al. (1974) first demonstrated that naloxone administered by itself produced hypernociceptive responses in rats; subsequently, some laboratories have confirmed this hypernociceptive action of naloxone in rodents (Grevert and Goldstein, 1977; Carmody et al., 1979). Goldstein et al. (1976) failed to observe the hypernociceptive naloxone effect. Frederickson et al. (1977) later reported that the hypernociceptive response produced by naloxone followed a diurnal rhythm.

In humans, Grevert and Goldstein (1978) showed that naloxone failed to alter responses in experimentally-induced pain situations namely cold water immersion and ischemia. Alternatively, Buschbaum et al., (1977) reported that naloxone produced hypoalgesia and hyperalgesia in individuals determined to be pain sensitive and pain insensitive, respectively. The diurnal variation in nociceptive respon-

siveness produced by naloxone in laboratory animals has also been demonstrated in humans (Davis et al., 1978). Finally, naloxone enhanced the post-operative pain arising from the removal of impacted molars (Levine et al., 1978). Naloxone also restored pain sensitivity, measured as an increase in flexor reflex, in an individual who was congenitally insensitive to pain (Dehen et al., 1978).

Overall, it appears that naloxone produces hypernociception and hyperalgesia only in special cases which may be associated with increased activity in the endogenous opioid system such as stress (Madden et al., 1977; Rossier et al., 1977a).

Several other pharmacologically- and physiologically-induced antinociceptive states can be antagonized by naloxone: included here are acupuncture (Mayer et al., 1976); transcutaneous electrical stimulation (Chapman and Benedetti, 1977); stimulation produced analgesia (SPA; Akil et al., 1976; see section 1.4.4 for further discussion); stress induced by cold water (Bodnar et al., 1977), footshock (Chesher and Chan, 1977), and immobilization (Amir and Amit, 1978); phenoxybenzamine (Elliot et al., 1976); nitrous oxide (Berkowitz et al., 1977); physostigmine and oxotremorine (Harris et al., 1969); acetylcholine (Pedigo et al., 1975); and diisopropyl phosphofluoridate (DFP; Koehn and Karczmar, 1978). It must be pointed out that naloxone antagonism of antinociception and analgesia is necessary but not sufficient evidence to conclude the involvement of the endogenous opioid system as mediating these effects. Since non-specific actions of

naloxone could contribute to its antagonism of antinociception and analgesia it is necessary to determine whether this antagonism is stereospecific.

#### 1.4.4 Stimulation Produced Analgesia (SPA)

Reynolds (1969) first demonstrated that focal electrical stimulation applied to the midbrain periaqueductal gray matter (PAG) rendered rats sufficiently antinociceptive to permit abdominal surgery. Since then, numerous studies have demonstrated that focal electrical stimulation of discrete brain regions produced a profound, long lasting level of antinociception (SPA) in laboratory animals as well as in humans (see section 1.1.8.3).

The most significant factor, however, concerning SPA is the fact that SPA and opioid-peptide-induced antinociception appear to share both common sites and mechanisms of action. A number of studies have mapped the brain for effective antinociceptive sites with SPA and opioid microinjections.

Electrical stimulation of the mesencephalic gray matter and periaqueductal grey matter produced antinociception as measured by a variety of noxious stimuli in the rat (Mayer and Liebeskind, 1974); loci in the central gray surrounding the aqueduct and caudal portions of the third ventricle were particularly effective (Rhodes and Liebeskind, 1978). Similarly, the most sensitive sites to intracerebral application of morphine in the rat lie in the ventrolateral sections of the



caudal PAG region (Yaksh et al., 1976b).

Electrical stimulation of other brain areas such as the septum, medial thalamus, ventral tegmentum, and pretectal area produced test specific antinociceptive states; ventrobasal thalamic and lateral hypothalamic sites were ineffective (Mayer and Liebeskind, 1974; Rhodes and Liebeskind, 1978). On the other hand, morphine injected into the posterior, anterior, and ventromedial hypothalamus, medial thalamus, and caudate increased flinch-jump thresholds in rats (Jacquet and Lajtha, 1973). Inejcted into the septum, morphine was ineffective (Jacquet and Lajtha, 1973).

The nucleus raphe magnus (NRM) of the medulla yields a profound level of antinociception following electrical stimulation in the rat and cat (Proudfit and Anderson, 1975; Oliveras et al., 1978; see section 1.3.4). Alternatively, the nucleus reticularis gigantocellularis of the medulla is extremely sensitive to local injections of morphine; applications into other medullary nuclei including NRM were ineffective (Takagi et al., 1977).

In summary, the degree of overlap for common effective brain sites of SPA and morphine, while not perfect, is impressive as it concerns the medial portions of the brain stem extending from the rostral medulla to the diencephalon. In fact, concurrent mapping studies performed in the same animal demonstrate that PAG sites are particularly sensitive to both procedures (Yeung et al., 1977).

The tail flick response in rats is considered to be a spinal reflex since both spinally intact animals as well as animals with spinal transections (T4-T5) respond to noxious heat stimulus applied to the rat's tail (Irwin et al., 1951). Since both morphine and SPA have been reported to block the tail flick response in rats (Mayer et al., 1975), the spinal cord appears to participate, at least in part, in mediating antinociception produced by these two methods.

In addition, recent electrophysiological investigations demonstrate that morphine injections as well as electrical stimulation of PAG sites specifically inhibited the responses of spinal cord wide dynamic range interneurons to noxious heat but not to innocuous mechanical stimuli. Neither the narcotics nor electrical stimulation affected neurons which responded exclusively to innocuous stimuli; thus, both procedures appear to specifically inhibit input at the spinal cord level (Bennett and Mayer, 1979).

Cross tolerance studies were performed to identify common neural substrates activated by SPA and morphine. Tolerance to SPA develops upon repeated stimulation in both the rat (Mayer and Hayes, 1975) and human (Hosobuchi et al., 1977); this tolerance exhibits cross tolerance to the antinociceptive and analgesic actions of morphine; tolerance to morphine reduced the effectiveness of SPA in the rat (Mayer, and Hayes, 1975) whereas tolerance to SPA increased the dose of morphine required to produce analgesia in humans (Hosobuchi et al., 1977). Tolerance to SPA and cross tolerance with morphine decreased after periods of nonstimulation in both species (Mayer and Hayes,

1975; Hosobuchi et al., 1977).

As stated earlier (see section 1.4.3), Akil et al. (1976) demonstrated that the narcotic antagonist, naloxone, reduced by approximately 30% the antinociceptive state produced by SPA applied to the PAG region in the rat brain. Other investigators demonstrated only a minor antagonistic effect of naloxone 3 to 5 minutes post stimulation (Pert and Walter, 1976) while still others report no effect of naloxone at all (Yaksh et al., 1976b). On the other hand, naloxone completely reversed the analgesia produced by PAG stimulation in 6 out of 7 patients studied (Adams, 1976; Hosobuchi et al., 1977).

Finally, analgesia arising from electrical stimulation of the PAG region was associated with increased amounts of enkephalin-like-material (Akil et al., 1978) and immunoreactive- $\beta$ -endorphin (Hosobuchi et al., 1979) in the ventricular cerebrospinal fluid (CSF); leucine-enkephalin levels were not altered (Hosobuchi et al., 1979).

Patients diagnosed as having organic pain exhibited significantly lower levels of methionine-enkephalin-like-material when compared with normal non painful volunteers or patients diagnosed as having psychogenic pain (Almay et al., 1978). In addition, patients with high levels ( $>0.9$  pmol/ml) of methionine-enkephalin-like-material exhibit higher pain thresholds and pain tolerance levels than did patients with low levels ( $<0.9$  pmol/ml) of methionine-enkephalin-like-material (Von Knorring et al., 1978).

In summary, although the exact role of the endogenous opioid system in antinociception and analgesia remains to be established, the combined data reported above suggest that these naturally occurring peptides may be involved in the control of pain.

## 2. RESEARCH OBJECTIVES

All the known potent naturally occurring and various synthetic narcotic analgesics produce tolerance and physical dependence (Isbell, 1977). In spite of the fact that no nonaddicting potent analgesic exists, the research devoted to this particular problem has led to the development of other useful drugs such as methadone which is used for withdrawal procedures and to maintain persons addicted to the opioids (Isbell, 1977). Another benefit has been the stimulation of the basic sciences to pursue research programs directly and indirectly related to the management of the chronic pain state. These research efforts have led to a more complete understanding of the anatomical, neurochemical, neurophysiological, and psychological mechanisms involved in the perception of pain.

Compounds which produce antinociception when administered to laboratory animals do not necessarily produce analgesia when administered to humans; however, a correlation between the antinociceptive and analgesic properties of a drug does exist for many narcotics (Isbell, 1977), certain compounds which affect the cholinergic system (Herz, 1961; Harris et al., 1968; Sitaram et al., 1977; Sitaram, 1979, personal communication), and various derivatives of delta-9-tetrahydrocannabinol (Bhargava, 1978a; Jochimsen et al., 1978). Thus, investigating drug-induced antinociception as an index of effectiveness of that drug to produce analgesia, while not applicable for all

drugs which produce antinociception is a valid procedure for specific drugs and/or classes of drugs.

Previous studies indicate that ACh (Pedigo et al., 1975), various cholinomimetics (Chen, 1958; Herz, 1961; Harris et al., 1967, 1968), and/or anticholinesterases (Harris et al., 1968; Bhargava and Way, 1972; Koehn and Karczmar, 1978) produce antinociception as well as potentiate narcotic-induced antinociception when administered to rodents. In addition, anticholinesterases, long known to potentiate narcotic-induced analgesia in humans (Floodmark and Wrammer, 1945) have recently been shown to produce analgesia when employed alone (Sitaram et al., 1977). Thus, present evidence suggests that compounds which affect the cholinergic system may be useful agents to investigate concerning the management of pain. The purpose of this dissertation research is to examine the antinociceptive property of the anticholinesterase agent, diisopropyl phosphofluoridate, DFP, and its various aspects.

The first objective of this research is to determine whether similarly to other anticholinesterases DFP will produce antinociception when administered to rats.

Anticholinesterase agents produce a state of immobilization (Adams, 1973) and hypothermia (Meeter and Wolthus, 1968) when administered to rats. Since the methods employed to assess antinociception, the hot plate and tail flick tests, involve motor and temp-

erature dependent behavioral responses it will be important to separate the antinociceptive effects from the attenuated mobility and hypothermia produced by DFP; exploratory and motor activity levels and rectal temperatures will be measured for this purpose.

The second objective of this research is to determine whether the antinociceptive action of DFP in the rat is related to the involvement of either the serotonergic or endogenous opioid systems in the CNS. Since increased activity of 5-HT neurons in the CNS is associated with the production of antinociception (see section 1.3) and DFP markedly increases 5-HT levels and turnover rates (Barnes et al., 1975, 1978), it may be speculated that DFP-induced antinociception results from actions involving the serotonergic system (see section 1.2.2).

On the other hand, an endogenous opioid system may mediate DFP-induced antinociception (see section 1.4). Several endogenous opioid peptides such as leucine- and methionine-enkephalin (Blasig and Herz, 1976) and  $\beta$ -endorphin (Szekely et al., 1977) produce antinociception when administered exogenously. Since antinociception produced by stress (Chesher and Chan, 1977 ; Bodnar et al., 1978), brain stimulation (Adams, 1976), acupuncture (Mayer et al., 1976), as well as a number of drugs (Elliot et al., 1976; Stewart et al., 1976; Harris et al., 1976; Berkowitz et al., 1977) is antagonized by naloxone, a narcotic antagonist, these various procedures may release endogenous opioids which then serve as endogenous antinociceptive agents. Nal-

oxone also antagonizes the antinociceptive state produced by physostigmine and oxotremorine (Harris, 1970) and ACh (Pedigo et al., 1975). Thus, if the enkephalins or endorphins indeed function as endogenous antinociceptive agents, then the antinociceptive state produced by DFP may result from DFP-induced release of the endogenous opioids.

It is hoped that the study of the mechanisms involved in DFP-induced antinociception may provide an explanation for certain conflicting findings and may also shed some light upon the mechanisms of antinociception in general. Finally, this investigation may, possibly, contribute to the management of pain by providing the rationale for implementing novel forms of drug therapy.



### 3. MATERIALS AND METHODS

#### 3.1 Animals

Male Sprague-Dawley rats, 160-350 gm obtained from Locke-Erickson and King Animal Distributors were used for behavioral studies as well as for brain serotonin (5-HT) and enkephalin level determinations. Male CF1 mice, 25-40 gm obtained from Charles River Animal Distributors were used for vasa deferentia bioassay preparations. Animals were housed in a temperature-controlled ( $22 \pm 2^{\circ}\text{C}$ ) room with lights on and off at 0700 and 1900 hours daily. In all cases, food and water were provided ad libitum.

#### 3.2 Drugs and Chemicals

##### 3.2.1 Source

Diisopropyl phosphofluoridate (DFP) obtained from K & K Laboratories, Plainview, New York in propylene glycol vehicle was diluted as appropriate in peanut oil (Mallinckrodt Chemical Company).

Morphine sulfate (Merck Chemical Division); atropine sulfate (City Chemical Company); atropine methyl nitrate, para-chlorophenyl-alanine methyl ester (PCPA) and pilocarpine HCl (Sigma Chemical Company); naloxone HCl (Endo Laboratories); MR 2266 and MR 2267 (- and + stereoisomers of 5,9  $\alpha$ -diethyl-2-(furylmethyl)-2'-hydroxy-

6,7-benzomorphan, Boehringer Ingelheim); GPA 1843 and GPA 1847 (- and + stereoisomers of 2-allyl-2'-hydroxy-9  $\beta$ -methyl-5-phenyl-6,7 benzomorphan, CIBA-Geigy Corporation); and d- and l-cyclazocine (Sterling-Winthrop Research Institute) were dissolved in normal saline.

Serotonin creatinine sulfate complex (Sigma Chemical Company) was dissolved in 0.1 N HCl (stock solution) and redissolved freshly each day in 0.1 N HCl for use in the fluorometric assay. Leucine- and methionine-enkephalin (Beckman Biochemicals) were dissolved in distilled water (stock solution) and redissolved in Krebs solution for use in the bioassay.

Distilled water, taken from the central storage tap, was de-ionized (Barnstead Ultrapure Mixed Bed) and redistilled (Corning Water Distillation Apparatus) for use throughout these experiments.

### 3.2.2 Dosage and Schedules

DFP (1.5, 1.0, 0.5, and 0.1 mg/kg) and morphine (3.0 mg/kg) were administered subcutaneously (s.c.) one hour prior to initiation of experiments. Atropine sulfate (4.0 mg/kg), atropine methyl nitrate (4.0 mg/kg), naloxone (5.0, 0.5 and 0.05 mg/kg), MR 2266 and MR 2267 (1.0 mg/kg), GPA 1843 and GPA 1847 (5.0 mg/kg), and d- and l-cyclazocine (0.64 mg/kg) were administered intraperitoneally (i.p.) 30 minutes prior to experimentation. Pilocarpine (2.5 mg/kg) was administered subcutaneously 30 minutes prior to experimentation.

PCPA was administered in doses of 100 (PCPA<sup>1</sup>) or 300 (PCPA<sup>2</sup>)

mg/kg i.p. on each of the two days preceeding testing; a third dose of 100 mg/kg i.p. was given two hours before the initiation of experiments.

In all cases, control animals were administered the drug vehicle, peanut oil or saline, in an identical manner.

### 3.2.3 Development of Tolerance

For the production of tolerance, animals were injected with morphine 3 times per day (at 0800, 1600, and 2400 hours) at a total daily dose as follows: 30, 60, 90, 120, 150, 180, 300, 450, and 600 mg/kg i.p. on days 1 through 9, respectively. Control animals were administered the vehicle. Hot plate responses (see section 3.3.2) were measured on day 6 (Tolerance level A) and on day 9 (Tolerance level B).

## 3.3 Behavioral Studies

### 3.3.1 Pretest Care of Animals

Rats were housed 3 or 4 per cage. Since handling produces stress in rats (Ader, 1968) and the handling involved in testing may affect the performance of rats not previously handled (Joffe and Levine, 1973), rats were handled for at least 5 minutes on each of the three days preceeding any testing situation to minimize stress effects.

Preinjection (baseline) measurements of all responses except exploratory and motor activity level determinations (see sections

4.2.1, 4.2.2, 4.6.1, 4.6.2, 4.7.2, and 4.7.3) were obtained at least one hour prior to the administration of any compound.

Mice were housed 10 per cage until their use for bioassay preparations.

### 3.3.2 Hot Plate Test

#### 3.3.2.1 Apparatus

The following two apparatuses were utilized for hot plate studies. First, inverted blocks of a Tecam Driblock DB-3 Test Tube Heater served as the hot plate source. The heat blocks were surrounded by a wire cage (23 cm long x 9.5 cm wide x 9.5 cm high) which restricted the animals' movement to the heat block surface. The temperature was maintained at  $55 \pm 0.5^{\circ}\text{C}$  by a variable heat control. Rats weighing 160-220 gm were employed in experiments concerning (a) effect of atropine sulfate and atropine methyl nitrate on DFP-induced antinociception (see section 4.1.3), (b) effect of PCPA on DFP-induced antinociception (see section 4.5.1), and (c) pharmacological comparison of DFP- and morphine-induced antinociceptive states (see section 4.7.1).

Second, a standard hot plate (32 cm square surface, property of the University of Iowa) was utilized. A plastic cylinder (22 cm diameter x 30 cm high) restricted the animals' movements to the hot plate surface. The temperature was monitored and maintained at  $55 \pm 0.5^{\circ}\text{C}$  by a series of built-in thermistors which provided the entire hot

plate surface with a constant temperature. Rats weighing 200-350 gm were employed in experiments concerning: (a) DFP- and pilocarpine-induced antinociception (see section 4.1.1), (b) Effect of naloxone on DFP-induced antinociception (see section 4.8.1), (c) Effect of stereoisomers of narcotic antagonists on DFP-induced antinociception (see section 4.9), and (d) Effect of tolerance to morphine on DFP- and pilocarpine-induced antinociception (see section 4.10).

#### 3.3.2.2 Testing Procedure

The hot plate test was carried out as follows. The rats were placed on the hot plate surface; the latency of onset of the following responses were recorded: (a) licking of the front paws, (b) licking or lifting/spinning movement of the hind paw, and (c) jumping off of the hot plate surface. All response times reported represent the latency of onset of the first response observed. A 30 second time limit was set to be the maximum nociceptive response time in all tests except those involving (a) DFP- and pilocarpine-induced antinociception (see section 4.1.1) and (b) Effect of naloxone on DFP-induced antinociception (see section 4.8.1) where a 60 second time limit was used.

#### 3.3.3 Tail Flick Test

In the tail flick test, animals were placed in a plastic restraining cage which allowed the tail to extend outwards and to relax for 3 minutes. The tail was then placed in a grooved slot of an asbestos

board; the beam of the light source, Blue M Infra Oven (375 watts), was focused one inch from the tip of the rats' tail. A 60 second time limit was set and the characteristic flicking movement of the tail was used as the endpoint.

### 3.3.4 Activity Level Determinations

#### 3.3.4.1 Apparatus

Exploratory and motor activity levels were assessed in a covered circular activity cage (Lehigh Valley Instruments Corporation) with a 46 cm high cylindrical wall. Six photoelectric cells, with opposing lights, were placed every 8.7 cm along the cylindrical wall 2.5 cm above the floor. Two counters, each responsive to 3 photoelectric cells, recorded the number of times each beam of light was broken.

#### 3.3.4.2 Exploratory and Motor Activity

The number of photoelectric interruptions were recorded 15 and 75 minutes after placing the rats individually in the activity cages. Activity levels reported represent the average of the two counters. Activity levels measured during the first 15 minutes and subsequent 60 minutes of the test are commonly referred to as the exploratory and motor activity periods, respectively (Karczmar and Scudder, 1967).

Exploratory activity levels were also measured by placing the animals individually in the activity cages for 10 minute periods at

at various times post drug administrations. Counters recorded the number of photoelectric interruptions during each 10 minute test session.

### 3.4 Rectal Temperature

Rectal temperatures in rats were measured by means of a thermister probe (Yellow Springs Instrument Company) inserted into the rectum to a depth of 50 mm and retained in situ until a constant temperature reading was obtained (usually 30 seconds).

### 3.5 Brain Serotonin (5-HT) Assay

Brain (5-HT) levels were determined according to modifications of methods originally described by Attack and Magnusson (1970) and Attack (1973). This experimental procedure utilizes the processes of cation-exchange chromatography to separate and spectrophotofluorometry to quantify 5-HT; it was employed with respect to 3 different brain regions, and can be outlined as follows:

#### 3.5.1 Tissue Samples

(a) Rats were sacrificed by decapitation (1000 to 1200 hours); brains were quickly removed from the cranial vault.

(b) Meninges and cerebellum were discarded.

(c) Brains were rapidly divided into three regions:

(1) Medullary (medulla)

(2) Meso-diencephalon (thalamus, hypothalamus, pons, and midbrain)

(3) Telencephalon (cortex and caudate)

(d) Brain regions were frozen in liquid nitrogen and stored at  $-20^{\circ}\text{C}$  until analyzed.

### 3.5.2 Reagents

(a) Water (see section 3.2.1)

(b) 2N NaOH + 1% EDTA (Dissolve 40 gm NaOH and 5 gm EDTA in 500 ml water)

(c) 10N, 2N, and 1N HCl (Made appropriately from concentrated 12 N HCl)

(d) ETOH/HCl (Combine equivalent volumes of redistilled 95% ETOH and 2N HCl)

(e) 0.1N Sodium Phosphate Buffer (Dissolve 8.83 gm  $\text{NaH}_2\text{PO}_4$ , 12.88 gm  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , and 1 gm EDTA in 1,000 ml water)

(f) Homogenizing Solution (Dissolve 0.125 gm  $\text{Na}_2\text{S}_2\text{O}_5$  and 0.5 gm EDTA in water. Add 8.55 ml 70%  $\text{HClO}_4$  and bring to a final volume of 250 ml with water)

(g) 5N  $\text{K}_2\text{CO}_3$  (Dissolve 69.0 gm  $\text{K}_2\text{CO}_3$  in 200 ml water)

(h) Brom Phenol Blue Solution (B.P.B.; dissolve 16 mg 3'3"5'5"-tetrabromophenolsulphonephthalein Na in 100 ml of redistilled 95% ETOH)

(i) Ortho-phthaldehyde (OPT, stock solution; dissolve 29.65 mg of OPT in 50 ml of 10N HCl)

OPT (experimental solution; take 8.72 ml of OPT stock solution up to 100 ml final volume with 10N HCl).



(j) Serotonin (5-HT, stock solution; dissolve 42.95 mg of serotonin creatinine sulfate complex in 250 ml of 0.1N HCl)

5-HT (Experimental solution; take 1.0 ml of 5-HT stock solution up to 50 ml final volume with 0.1N HCl)

### 3.5.3 Column Preparation

#### 3.5.3.1 Cycling and Regeneration of Resin

Ion-exchange chromatography resin should be given at least one complete change of form to remove other ions and impurities (Mikes, 1970). Utilizing a magnetic stirring apparatus, 250 gm of DOWEX AG 50W X4 200-400 mesh H<sup>+</sup> from ion-exchange resin was cycled and regenerated ten times with the following solutions:

(a) 300 ml 2N NaOH + 1% EDTA

(b) 300 ml ETOH/HCl

(c) 300 ml 2N HCl

(d) 300 ml water

The regenerated resin was stored in water at 5°C.

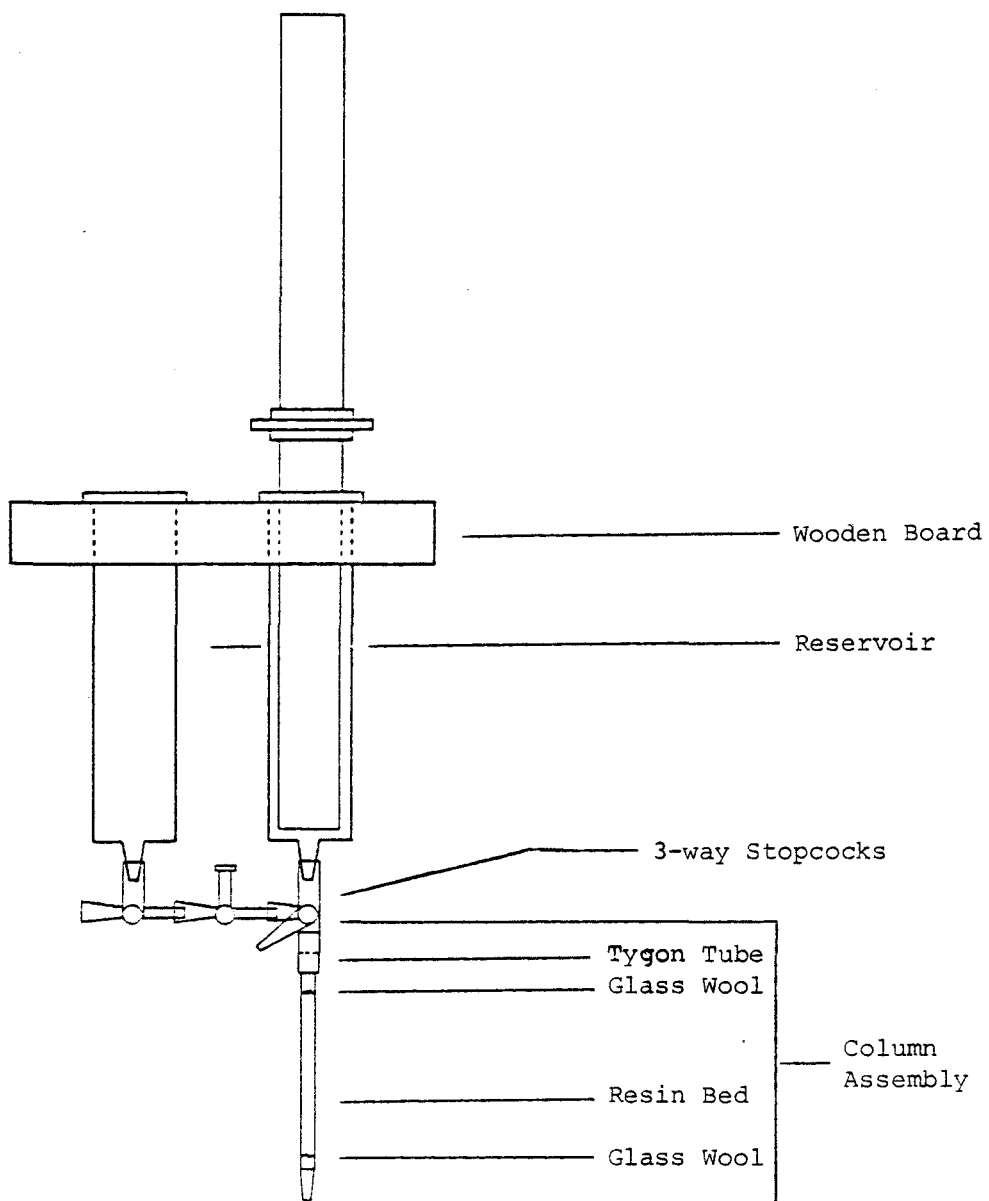
#### 3.5.3.2 Packing of Columns

The columns to be packed with ion-exchange resin have been adapted from 1.0 ml disposable syringes (4.2 mm internal diameter x 7.0 cm high; Fig. 1). A piece of tygon tubing, which serves to attach the column assembly to the glass syringe apparatus was affixed to the upper end of the syringe barrel (Fig. 1). Since air bubbles disrupt the uniform packing of the resin, the actual packing was performed

## FIGURE 1

Diagram of column assembly and glass syringe apparatus

Fig. 1



with the columns submerged in water. First, a small piece of glass wool which serves to trap the resin in the column, was inserted into the column and forced to the tip with a stirring rod (Fig. 1). A 2 ml pipet filled with resin was inserted into the top of the column and the resin was allowed to flow into the column. When the resin bed had reached a height of 50 mm, another piece of glass wool was inserted into the top of the column, thus trapping the resin between the two pieces of glass wool (Fig. 1). The freshly packed column was mounted onto one of the glass syringes in the glass syringe apparatus (Fig. 1). The glass syringe apparatus consisted of the following. Two 20 ml glass syringe barrels were connected to each other through a series of three-way stopcocks (Fig. 1). One syringe barrel served as a reservoir through which the extract and other reagents could be added to the column (Fig. 1). The other glass syringe barrel served as the site of attachment for the column (Fig. 1). The glass syringe apparatus was suspended vertically through holes in a wooden board (Fig. 1).

#### 3.5.3.3 Preparing Columns for Samples

It is often necessary to ion exchange resin for elution ion-exchange chromatography to adjust the pH to optimal for separation (Mikes, 1970). Here, the resin was prepared for the addition of tissue extracts by cycling the resin into the form of the buffer cation ( $\text{Na}^+$ ) and then washing with buffer (Atack, 1973). This was accomplished by passing the following solutions through the column: (a) 20 ml 2N NaOH + 1% EDTA, (b) 50 ml water, (c) 20 ml 2N HCl, (d) 50 ml water,

(e) 20 ml 0.1N sodium phosphate buffer (pH=6.5), and (f) 5 ml water. The pH of the effluent is taken as a control measurement to check that the inflowing solution and effluent have the same composition; thus, a pH of 6.5 indicated that the column was ready for the addition of the tissue extracts.

#### 3.5.4 Extraction Process

(a) Weighed brain regions were chilled and homogenized in 8.0 ml of homogenizing solution (see section 3.5.2).

(b) Homogenate was centrifuged at 4000 RPM for 15 minutes.

(c) Supernatant was poured into 15 ml graduated tubes.

(d) Pellet was reextracted with 3.0 ml of homogenizing solution; centrifuged at 4500 RPM for 10 minutes; supernatants were combined.

(e) pH was adjusted to 6.5 with 5N  $K_2CO_3$  (using 2 drops of B.P.B. as pH indicator).

(f) Supernatant was set for 10 minutes on ice.

(g) Centrifuged at 5000 RPM for 15 minutes.

(h) Tissue extracts filtered (Watman No.1) onto ion-exchange column; passed through column (flow rate <0.5 ml/min) mol.

(i) Columns rinsed by passing the following solutions:

(1) 15 ml water

(2) 15 ml 0.1N sodium phosphate buffer

(3) 15 ml water

(j) 5-HT was eluted with the following solutions:

(1) 10.5 ml 1N HCl (discard)

(2) 4.7 ml ETOH/HCl (discard)

(3) 6.0 ml ETOH/HCl (collect = 5-HT fraction)

Internal standards were prepared by substituting 1400, 700, and 350 ng of 5-HT as well as 0.5 ml of ETOH and H<sub>2</sub>O in place of a brain sample. Per cent recovery of 5-HT and column reagent blanks fluorescent values were determined from these five internal standards.

### 3.5.5 Conversion of Serotonin (5-HT) to Fluorophore

Serotonin (5-HT) content in biological tissues can be determined by fluorescent methods directly (Maicker and Miller, 1966). However, reacting 5-HT with o-phthaldehyde (OPT) yields a substituted indole compound which is 20 times more fluorescent than 5-HT itself (sensitive to 1.0 ng 5-HT; Maicker and Miller, 1966). Therefore, 5-HT was reacted with OPT according to the methods described by Maicker and Miller (1966). The process is outlined as follows: (a) Add 2 drops of OPT and 0.5 ml of sample eluate to 1.0 ml 10N HCl (b) Boil for 15 minutes (c) Allow to cool to room temperature.

External standards and reagent blanks were prepared as follows. To 1.0 ml of 10N HCl the following amounts of standard 5-HT and reagents were added:

- (a) 700 ng 5-HT [0.500 ml 5-HT (Experimental solution) + 0.000 ml 0.1N HCl]
- (b) 350 ng 5-HT [0.250 ml 5-HT (Experimental solution) + 0.250 ml 0.1N HCl]

(c) 175 ng 5-HT [0.125 ml 5-HT (Experimental solution) +  
0.375 ml 0.1N HCL]

(d) ETOH Blank (0.500 ml ETOH)

(e) H<sub>2</sub>O Blank (0.500 ml H<sub>2</sub>O)

All samples and standards were read in an Aminco-Bowman Spectrophotofluorometer at uncorrected wavelengths of 360 mμ (activation) and 470 mμ (emission). The water reagent blank was set at 0.05 fluorescent units and the samples were read accordingly.

### 3.5.6 Calculations

The formula for calculating ng 5-HT/gm brain tissue is as follows:

$$\frac{A \times (B - C)}{(D - E) \times F \times G} = \text{ng 5-HT/gm brain tissue}$$

where,

A = 12 (6.0 ml/0.5 ml; total volume of 5-HT eluate fraction collected,  
see section 3.5.4

Volume of 5-HT eluate fraction converted to fluorophore, see  
section 3.5.5)

B = Fluorescent units of sample (tissue extract)

C = Fluorescent units of column reagent blank (determined from internal standards passed through the column, see section 3.5.4).

D = Fluorescent units of external standard

E = Fluorescent units of external reagent blanks

F = Per cent recovery (determined from internal standards passed  
through the column, see section 3.5.4)

G = Brain region weight

Average recoveries for 5-HT were found to vary between 65 and 80 per cent. Brain sample concentrations (ng/gm) were always corrected for 100 per cent recovery based on the internal standard recovery percentage for each experiment.

### 3.6 Brain Enkephalin-like-material Level Assay

Brain enkephalin-like-material levels were determined according to methods originally described by Hughes et al. (1975b; 1977) and modified at present. Experimental procedures involved are (1) molecular sieve chromatography to separate and (2) vas deferens bioassay preparation to quantify enkephalin-like-material; procedures are outlined as follows.

#### 3.6.1 Tissue Samples

(a) Rats were sacrificed by decapitation (0800 to 1000 hours); brains were quickly removed on ice.

(b) Meninges and cerebellum were discarded.

(c) Weighed brains were placed in 10 ml of 0.1 N HCl for the extraction of enkephalin-like-material (see section 3.6.4).

#### 3.6.2 Reagents

(a) Water (see section 3.2.1)

(b) 0.1N HCl (Made appropriately from concentrated 12N HCl)

(c) Dichloromethane (As obtained commercially)



(d) Methanol (MeOH) (Degassed under vacuum in heavy walled filtering flask)

(e) Kreb's Solution (Dissolve 13.72 gm NaCl, 0.70 gm KCl, 0.734 gm  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.332 gm  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 3.96 gm d-glucose, and 4.20 gm  $\text{NaHCO}_3$  in 2000 ml water)

(f) Leucine/Methionine-Enkephalin (Stock solution; dissolve 1.0 mg leucine/methionine-enkephalin in 1.0 ml water)

(g) Leucine/Methionine-Enkephalin (Experimental solution; take 10  $\mu\text{l}$  of leucine/methionine-enkephalin stock solution up to a final volume of 10 ml in Kreb's solution)

### 3.6.3 Column Preparation

#### 3.6.3.1 Resin Cleaning Procedure

Chromatography resin was cleaned prior to its use to remove any impurities. Utilizing a magnetic stirring apparatus, 250 gm of Amberlite XAD2, neutral polystyrene bead resin was washed with the following solutions ten times:

- (a) 300 ml Dichloromethane
- (b) 300 ml Isopropyl alcohol
- (c) 300 ml water

The cleaned resin was stored in water at room temperature.

#### 3.6.3.2 Packing of Columns

Cylindrical columns (25 cm high, Kontes Martin Company) were used in this procedure. The lower portion of the column (14 cm, 8.0

8.0 mm internal diameter) held the chromatography resin; the upper portion (11 cm, 32 mm internal diameter; 125 ml capacity) served as a reservoir for the application of brain extracts and various solutions. A rotating plastic stopcock located at the base of the column adjusted the flow rate. The columns were suspended vertically through holes in a wooden board. A small piece of glass wool was inserted into the column to trap the resin at the lower end. The column was filled with resin to a height of 80 mm. To remove air bubbles which interfere with the uniform packing of the resin and flow of solutions through the columns, a small glass stirring rod was used periodically during the times at which the columns were filled with resin as well as during the subsequent extraction process.

#### 3.6.4 Extraction Process

- (a) Chilled homogenization of weighed brains in 10.0 ml of 0.1N HCl
- (b) Centrifugation of homogenate in a total volume of 20-30 ml of 0.1N HCl at 19,000 RPM for 30 minutes
- (c) Pouring of supernatant onto prewashed (300 ml H<sub>2</sub>O) column and pass through resin (flow rate < 1 ml/minute)
- (d) Rinsing of columns by passing the following solutions:
  - (1) 40 ml 0.1N HCl
  - (2) 50-200 ml H<sub>2</sub>O
- (e) Eluting enkephalin-like-material with 40 ml MeOH (Degassed)
- (f) Vaporizing and withdrawing MeOH down to approximately 2 ml under vacuum at 40°C (Buchi Rotorvapor-R Apparatus)

(g) Transfer of remaining eluate to 25 ml round bottom flask and desiccation to complete dryness under vacuum at 50°C (Buchi Roto-vapor-R-apparatus)

(h) Freezing (-20°C) dried sample until used in bioassay

Internal standards were prepared by substituting 400 mg of leucine-enkephalin in place of two brain samples; per cent recovery of enkephalin for each experiment was determined from these two internal standards.

### 3.6.5 Bioassay

#### 3.6.5.1 Preparation of Vasa Deferentia

(a) Mice were sacrificed by cervical dislocation.

(b) Vasa deferentia were quickly removed and placed in Kreb's solution.

(c) Cotton threads were attached to one end of each vas deferens.

(d) Tissues were mounted vertically in a 1.7 ml organ bath (Kontes Martin Corporation, as by design) containing oxygenated (95% O<sub>2</sub>; 5% CO<sub>2</sub>); Krebs solution at 37°C.

(e) The upper end of each tissue was attached by a thread to an isometric or isotonic transducer (Harvard Apparatus).

(f) The tissue was placed initially under 200-500 mg tension.

#### 3.6.5.2 Electrical Stimulation

(a) Two platinum wires (10 mm long) located at each vertical

extremity served as stimulating electrodes.

(b) Tissues were submaximally stimulated with square pulses of 0.5 to 0.8 msec duration from a Grass S48 Stimulator.

(c) Frequencies of 0.07 to 0.10 Hz were used.

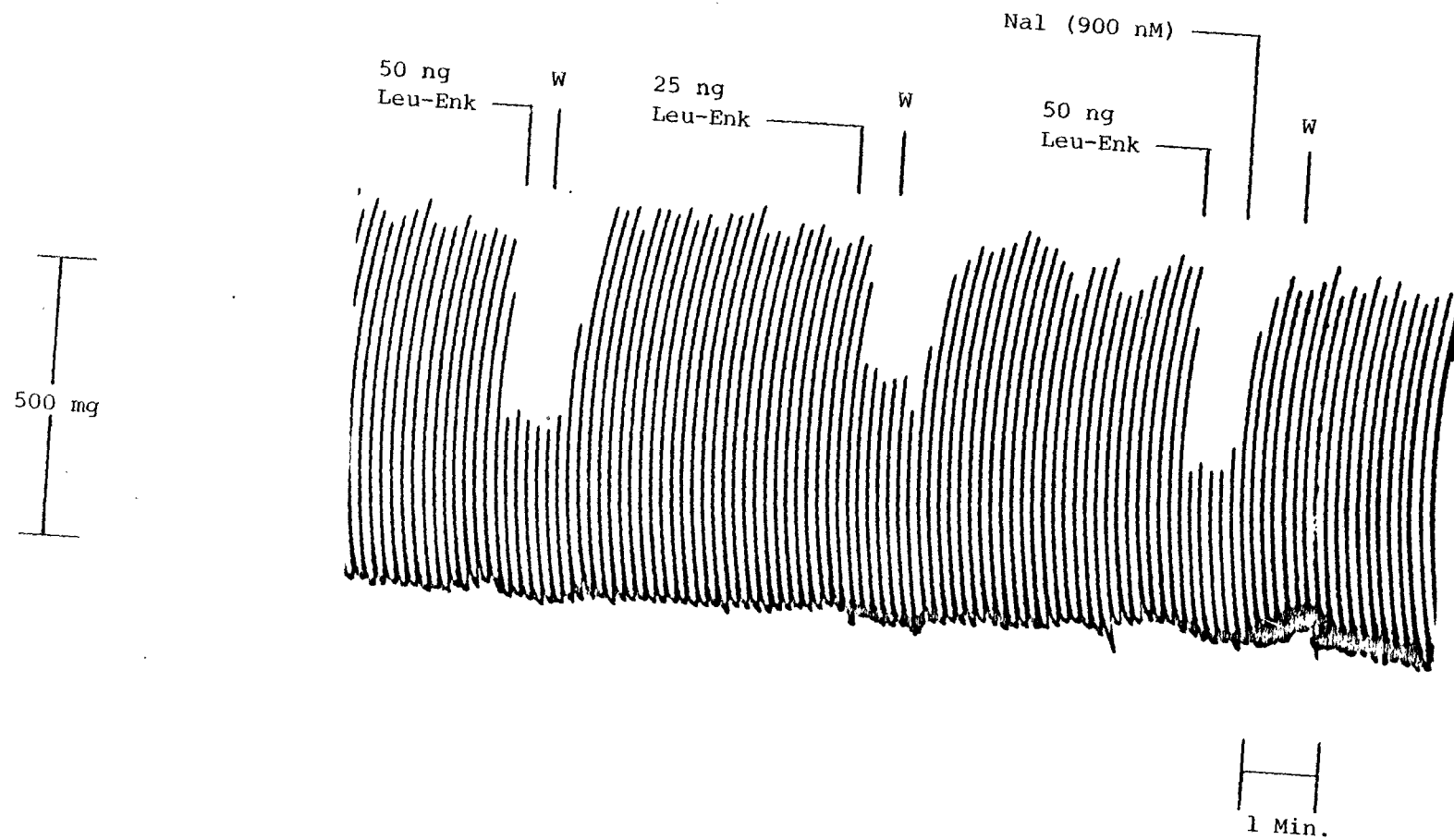
#### 3.6.5.3 Measurement of Enkephalin-like-material

Enkephalins inhibit the electrically evoked contractions of the longitudinal muscle of the mouse vas deferens by depressing evoked norepinephrine output; this effect is mediated through the opiate receptor (Hughes et al., 1975b). The depression of evoked contraction is represented as a depression of the twitch height and is related to the amount of enkephalin added to the bath in a dose dependent manner (Fig. 2). Utilizing a bracket assay, the amount of enkephalin-like-material in a sample may be determined as follows. First, constant responses ( $\pm 5\%$ ) to repeated applications of known amounts of standard leucine-enkephalin were obtained to insure reliability of tissue responses (Fig. 2). Second, various amounts of unknown sample are applied until an amount which depresses the twitch height by approximately 50% is determined. Third, the unknown sample is bracketed between predetermined low and high doses of standard leucine-enkephalin; the low dose of standard should depress the twitch height less than the brain sample; the high dose of standard should depress the twitch height more than the brain sample. Finally, complete antagonism, that is 100% reversibility, by the narcotic antagonist, naloxone (900 nM) of the twitch height depression due to the administration of the brain

## FIGURE 2

Inhibition of electrically evoked contractions of the longitudinal muscle of the mouse vas deferens by leucine-enkephalin (Leu-Enk). W = washout time. Application of naloxone (Nal. 900 nM, bath concentration) prior to washout is used to demonstrate that the depressant action of Leu-Enk is mediated through the opiate receptor.

Fig. 2



sample identified the depressant action of the brain sample as being mediated through the opiate receptor. A 6 minute dose cycle was used throughout.

### 3.6.6 Calculations

The amount of enkephalin-like material expressed as ng leucine-enkephalin-equivalents/gm brain tissue was calculated using the following formula:

$$\frac{A \times B}{C \times D \times E} = \text{ng leucine-enkephalin-equivalents/gm brain tissue}$$

where,

A = ng leucine-enkephalin equivalent in organ bath (determined from the standard curve drawn from the low and high doses of standard leucine-enkephalin used in the bracket assay)

B = Total volume of Krebs solution used to redissolve sample

C = Volume of sample injected into the organ bath

D = Per cent recovery (determined from internal standards, see section 3.6.4)

E = Brain weight

Average per cent recovery for internal standards of leucine-enkephalin (400 ng) varied between 20 and 90%. Calculations were always corrected for 100% recovery based on the internal standard recovery percentage for each experiment (see section 3.6.4).

### 3.7 Statistical Analysis

Hot plate responses (see sections 4.1.1, 4.1.3, 4.5.1, 4.7.1, 4.8.1, 4.9, and 4.10), tail flick responses (see sections 4.1.2, and 4.5.2), and rectal temperature measurements (see section 4.3 and 4.8.3) were compared to preinjection, baseline, values for each animal (paired student t test, two tailed).

Exploratory activity levels (see sections 4.2.2, 4.6.1, 4.7.2, and 4.8.2), motor activity levels (see sections 4.2.1, 4.6.2, and 4.7.3), brain region 5-HT levels (see section 4.4.1, 4.4.2, and 4.4.3) and whole brain enkephalin-like-material levels (see section 4.11) were compared to responses and values obtained from animals administered the appropriate vehicle/s (analysis of variance, Newman-Keuls test). Other tests for statistical significance are as stated in Chapters 4 and 5.

### 3.8 Definitions

The statistical analysis employed in this dissertation require more restrictive definitions of some commonly used terms as follows.

"Antinociception" is defined as statistically significant ( $p < 0.05$ ) increase in hot plate or tail flick response times (latencies) in rats administered a compound when compared to preinjection, baseline responses (paired student t test).

When the preinjection, baseline, and postinjection hot plate or tail flick response latencies (times) in rats administered known anti-



nociceptive doses of DFP or morphine did not differ ( $p > 0.05$ ; paired student t test) due to the administration/s of an additional compound, this effect was referred to as "antagonism of the antinociceptive state".

Rarely, the administration of a compound reversed the effect of known antinociceptive doses of DFP or morphine; that is, DFP or morphine in the presence of an additional compound produced a statistically significant ( $p < 0.05$ ) decrease in the latency of the hot plate or tail flick responses in rats when compared to preinjection, baseline responses (paired student t test). This effect is referred to as "reversal of the antinociceptive state".

Some compounds, administered alone, significantly decrease ( $p < 0.05$ ) the animals' hot plate or tail flick response latencies when compared to preinjection, baseline responses (paired student t test). This effect is referred to as the production of a "hypernociceptive state".

All other comparative terms such as increased, decreased, attenuated, etc. reflect statistically significant ( $p < 0.05$ ) differences as described by the appropriate statistical analysis.

#### 4. RESULTS

##### 4.1 DFP- and Pilocarpine-Induced Antinociception in Rats

###### 4.1.1 Hot Plate Test

The effect of a single s.c. administration of DFP (0.1, 0.5, 1.0, and 1.5 mg/kg) or pilocarpine (2.5 mg/kg) on the hot plate response times of rats was measured 1, 2, 4, 6, 8, 24, and 48 hours after the DFP and 30 minutes after the pilocarpine injection, respectively. DFP produced antinociception 1 hour post injection at all doses tested; pilocarpine (2.5 mg/kg) also produced a significant antinociceptive state 30 minutes after its administration; the vehicle was ineffective (Table 1). Response times of animals administered the vehicle were not significantly different when compared to response times of control, non-injected, animals in the hot plate test or in any other test or measurement described in this dissertation (analysis of variance, Newman-Keuls test; Table 1).

The antinociceptive state produced by DFP followed a dose dependent relationship in terms of potency and duration of effect. Thus, the response latency was increased by 77 to 94% by doses of 1.0 and 1.5 mg/kg of DFP, and by some 28% by the dose of 0.1 mg/kg. Antinociception produced by DFP (1.0 and 1.5 mg/kg) was maintained for 6 hours with maximum effects at 2 and 4 hours, respectively; DFP (0.1 and 0.5 mg/kg) produced antinociception that lasted for 1 hour after administration (Fig. 3). Finally, it should be noted that animals admin-

TABLE 1

Hot plate response times (seconds  $\pm$  S.E.M.) of rats subsequent to the single s.c. administration of the vehicle, DFP (0.1, 0.5, 1.0 and 1.5 mg/kg), or pilocarpine (2.5 mg/kg). n = number of animals employed in each group. Postinjection responses were compared to preinjection responses for each animal (paired Student's t Test). \* $p < 0.05$  and \*\* $p < 0.01$ .

TABLE 1

EFFECT OF DFP AND PILOCARPINE ON HOT PLATE RESPONSETIMES OF RATS

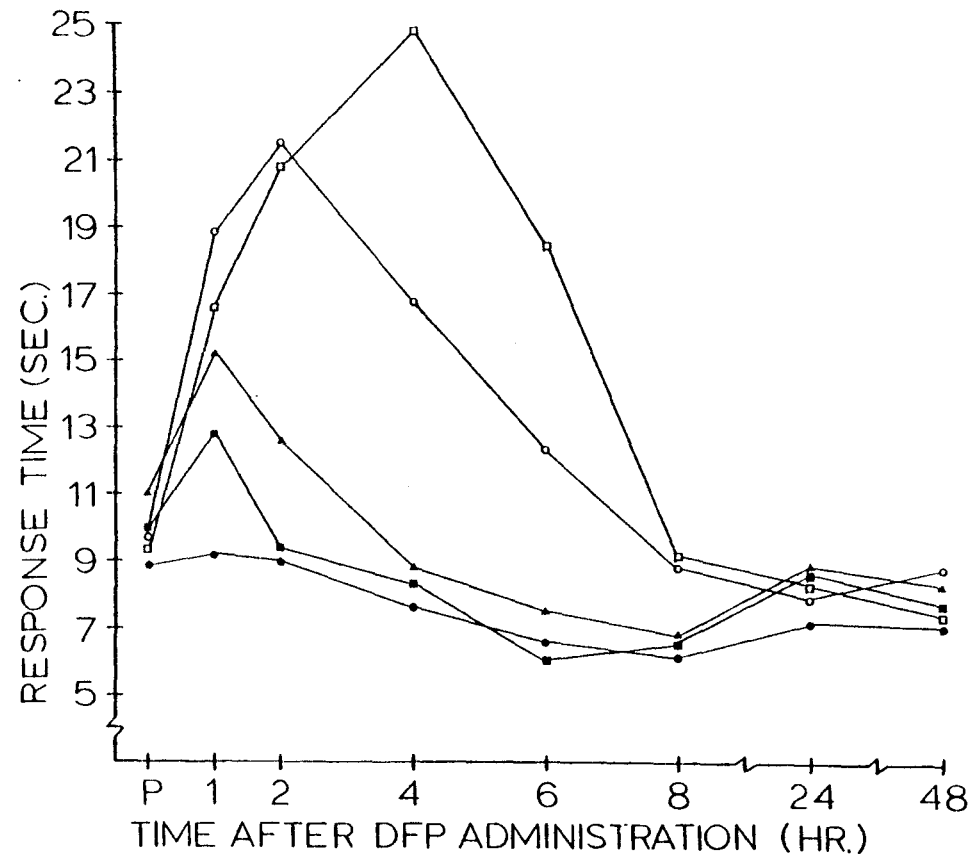
<u>Group</u>	<u>(n)</u>	<u>Hot Plate</u>	
		<u>Response Times (Sec) <math>\pm</math> S.E.M.</u>	
		<u>Preinjection</u>	<u>Postinjection</u>
Control (non-injected)	3	9.6 $\pm$ 0.7	9.5 $\pm$ 0.8
Peanut oil	8	8.9 $\pm$ 0.6	9.2 $\pm$ 0.6
DFP (0.1 mg/kg)	8	9.9 $\pm$ 0.9	12.8 $\pm$ 1.6*
DFP (0.5 mg/kg)	8	11.0 $\pm$ 0.9	15.2 $\pm$ 1.3*
DFP (1.0 mg/kg)	8	9.7 $\pm$ 0.9	18.8 $\pm$ 3.2**
DFP (1.5 mg/kg)	8	9.4 $\pm$ 0.8	16.6 $\pm$ 2.0**
Pilocarpine (2.5 mg/kg)	8	9.2 $\pm$ 1.3	16.0 $\pm$ 1.4**

\*p<0.05 and \*\*p<0.01

FIGURE 3

Hot plate response times (seconds  $\pm$  S.E.M.) of rats subsequent to the administration of vehicles (closed circles) or DFP (0.1 mg/kg, closed squares; 0.5 mg/kg, closed triangles; 1.0 mg/kg, open circles; and 1.5 mg/kg, open squares). Each point represents the mean  $\pm$  S.E.M. of eight animals. Hot plate response times were compared to preinjection (P on abscissa) response values (paired Student's *t* Test). Statistical analysis for significance is as stated in the text (see section 4.1.1).

Fig. 3



istered the vehicle or DFP (0.1 mg/kg) exhibited attenuated hot plate responses 6 and 8 hours postinjection (Fig. 3).

#### 4.1.2 Tail Flick Test

The effect of DFP (0.1, 0.5, 1.0, and 1.5 mg/kg) on the tail flick response times of rats was measured 1 hour after the DFP administration. DFP in doses of 1.5 and 1.0 mg/kg produced antinociception; at doses of 0.1 and 0.5 mg/kg, DFP was ineffective; animals administered the vehicle did not exhibit antinociception (Table 2).

#### 4.1.3 Effect of Atropine Sulfate and Atropine Methyl Nitrate

To determine whether DFP-induced antinociception arises from stimulation of central cholinergic receptors (see section 1.2.2), the effect of atropine sulfate and atropine methyl nitrate on DFP-induced antinociception was studied utilizing the hot plate test. Administered 30 minutes after the administration of DFP (1.5 mg/kg), atropine sulfate (4.0 mg/kg) antagonized whereas atropine methyl nitrate (4.0 mg/kg) did not affect the antinociceptive state produced by DFP (Table 3). Atropine sulfate (4.0 mg/kg) or the vehicles did not affect the hot plate response when administered alone (Table 3).

Altogether, it is obvious that, similarly to other drugs which affect the central cholinergic system, DFP and pilocarpine produce antinociception.

TABLE 2

Tail flick response times (seconds  $\pm$  S.E.M.) of rats subsequent to the single s.c. administration of the vehicle or DFP in doses of 0.1, 0.5, 1.0 and 1.5 mg/kg. n = number of animals employed in each group. Post-injection responses were compared to preinjection responses for each animal (paired Student's t Test). \*p<0.05.



TABLE 2

EFFECT OF DFP ON TAIL FLICK RESPONSE TIMES OF RATS

<u>Group</u>	<u>(n)</u>	<u>Tail Flick</u>	
		<u>Response Times (sec) <math>\pm</math> S.E.M.</u>	
		<u>Preinjection</u>	<u>Postinjection</u>
Control (non-injected)	8	17.9 $\pm$ 2.0	16.9 $\pm$ 1.5
Peanut oil	9	18.1 $\pm$ 1.9	18.1 $\pm$ 1.7
DFP (0.1 mg/kg)	8	17.7 $\pm$ 1.6	18.9 $\pm$ 2.4
DFP (0.5 mg/kg)	6	18.1 $\pm$ 2.1	18.3 $\pm$ 2.7
DFP (1.0 mg/kg)	6	18.0 $\pm$ 1.8	22.8 $\pm$ 2.6*
DFP (1.5 mg/kg)	8	17.2 $\pm$ 1.9	23.6 $\pm$ 1.8*

\*p &lt; 0.05

TABLE 3

Hot plate response times (seconds  $\pm$  S.E.M.) of rats subsequent to the administrations of vehicles, DFP (1.5 mg/kg)-saline, DFP (1.5 mg/kg)-atropine sulfate (4.0 mg/kg), DFP (1.5 mg/kg)-atropine methyl nitrate (4.0 mg/kg), and peanut oil-atropine sulfate (4.0 mg/kg). n = number of animals employed in each group. Post-injection responses were compared to preinjection responses for each animal (paired Student's t Test). \*\*p < 0.01 and \*\*\*p<0.001.

TABLE 3

EFFECT OF DFP ON HOT PLATE RESPONSE TIMES OF RATS

<u>Group</u>	<u>(n)</u>	<u>Hot Plate</u>	
		<u>Response Times (Sec) <math>\pm</math> S.E.M.</u>	
		<u>Preinjection</u>	<u>Postinjection</u>
Control (non-injected)	12	8.2 $\pm$ 0.3	7.8 $\pm$ 0.7
Peanut oil-saline	10	8.7 $\pm$ 0.5	9.1 $\pm$ 0.8
DFP (1.5 mg/kg)-saline	10	8.2 $\pm$ 0.3	16.9 $\pm$ 0.8***
DFP (1.5 mg/kg)-atropine sulfate (4.0 mg/kg)	8	8.4 $\pm$ 0.4	9.0 $\pm$ 0.5
DFP (1.5 mg/kg)-atropine methyl nitrate (4.0 mg/kg)	8	8.6 $\pm$ 0.4	14.9 $\pm$ 0.5**
Peanut oil-atropine sulfate (4.0 mg/kg)	6	7.9 $\pm$ 0.4	9.5 $\pm$ 0.4

\*\*p<0.01 and \*\*\*p<0.001

## 4.2 DFP-induced Immobilization in Rats

An established effect of DFP is reduction of the animal's motor activity (Karczmar, 1977). Since the effects of DFP on rat motor activity and environmental response may interfere with the hot plate assessment of antinociception, motor and exploratory activity levels were studied.

### 4.2.1 Exploratory Activity

The animal's exploratory activity, that is, its response to a novel environment may be a particularly pertinent correlate of antinociception as measured by the hot plate test.

The effect of DFP administered in doses of 0.1, 0.5 and 1.5 mg/kg on exploratory activity was studied one hour after the administration of DFP. DFP (1.5 mg/kg) decreased by 45% exploratory activity; in doses of 0.1 and 0.5 mg/kg, DFP was ineffective; administration of the vehicle did not affect exploration (Table 4).

To further investigate the effect of DFP on exploration, exploratory activity levels were measured 1 to 48 hours after the administration of DFP (0.1, 0.5, 1.0, and 1.5 mg/kg) at the same time intervals at which hot plate responses were measured (see section 4.1.1). DFP (1.5 mg/kg) reduced exploratory activity in rats 1, 2, and 48 hours after the DFP administration. DFP in doses of 0.1, 0.5, and 1.0 mg/kg reduced exploratory activity 2 hours after the DFP injection; the diminution in exploration at 2 hours followed a dose dependent

TABLE 4

Exploratory and motor activity levels (activity cage counts  $\pm$  S.E.M.) of rats subsequent to the administration of vehicle or DFP in doses of 0.1, 0.5 and 1.5 mg/kg. n = number of animals employed in each group. Exploratory and motor activity levels were compared to levels obtained from animals administered the vehicle (analysis of variance, Newman-Keuls test). \* $p < 0.05$  and \*\* $p < 0.01$ .

TABLE 4

EFFECT OF DFP ON EXPLORATORY AND MOTOR ACTIVITY LEVELSOF RATS

<u>Group</u>	<u>(n)</u>	<u>Activity Cage Counts <math>\pm</math> S.E.M.</u>	
		<u>Exploratory</u>	<u>Motor Activity</u>
Control (non-injected)	12	462 $\pm$ 27	511 $\pm$ 19
Peanut oil	9	448 $\pm$ 12	512 $\pm$ 12
DFP (0.1 mg/kg)	7	410 $\pm$ 33	404 $\pm$ 29**
DFP (0.5 mg/kg)	8	336 $\pm$ 28	239 $\pm$ 24**
DFP (1.5 mg/kg)	7	252 $\pm$ 19**	164 $\pm$ 13**

\*p<0.05 and \*\*p<0.01

relationship for all doses of DFP tested (Fig. 4). Exploratory activity levels were compared to levels obtained from animals administered the vehicle (analysis of variance, Newman-Keuls test; Fig. 4).

#### 4.2.2 Motor Activity

The effect of DFP administered in doses of 0.1, 0.5 and 1.5 mg/kg reduced motor activity levels (Table 4). The diminution in motor activity produced by DFP (0.1 to 1.5 mg/kg) followed a dose dependent relationship. Administration of the vehicle did not affect motor activity (Table 4).

#### 4.3 Effect of DFP on Rectal Temperature of Rats

Mild hypothermia (a 2 to 3°C decrease in rectal temperature below normal) produced hypernociception in the cat (Liu and Fong, 1972). Since the systemic administration of DFP produces hypothermia in the rat (Meeter and Wolthius, 1968), this effect of DFP may interfere with the correct evaluation of antinociception as measured by temperature sensitive tests such as the hot plate and tail flick tests. The effect of DFP on rectal temperatures of rats was measured 1 to 48 hours after the administration of DFP at the same time intervals at which hot plate responses were measured (see section 4.1.1). DFP (1.0 mg/kg) produced hypothermia (a 2.4°C decrease in rectal temperature) 2 hours postinjection at 4 hrs the effect was not significant any more. The vehicle was ineffective (Fig. 5).

FIGURE 4

Exploratory activity levels of rats expressed as the percent of preinjection activity cage counts  $\pm$  S.E.M. subsequent to the administration of vehicle (closed circles) and DFP (0.1 mg/kg, closed squares; 0.5 mg/kg, closed triangles; 1.0 mg/kg, open squares; and 1.5 mg/kg, open circles). Each point represents the mean of five animals. Exploratory activity levels were compared to levels obtained from animals administered the vehicle (analysis of variance, Newman-Keuls test). Statistical analysis for significance is as stated in the text (see section 4.2.2).



Fig. 4

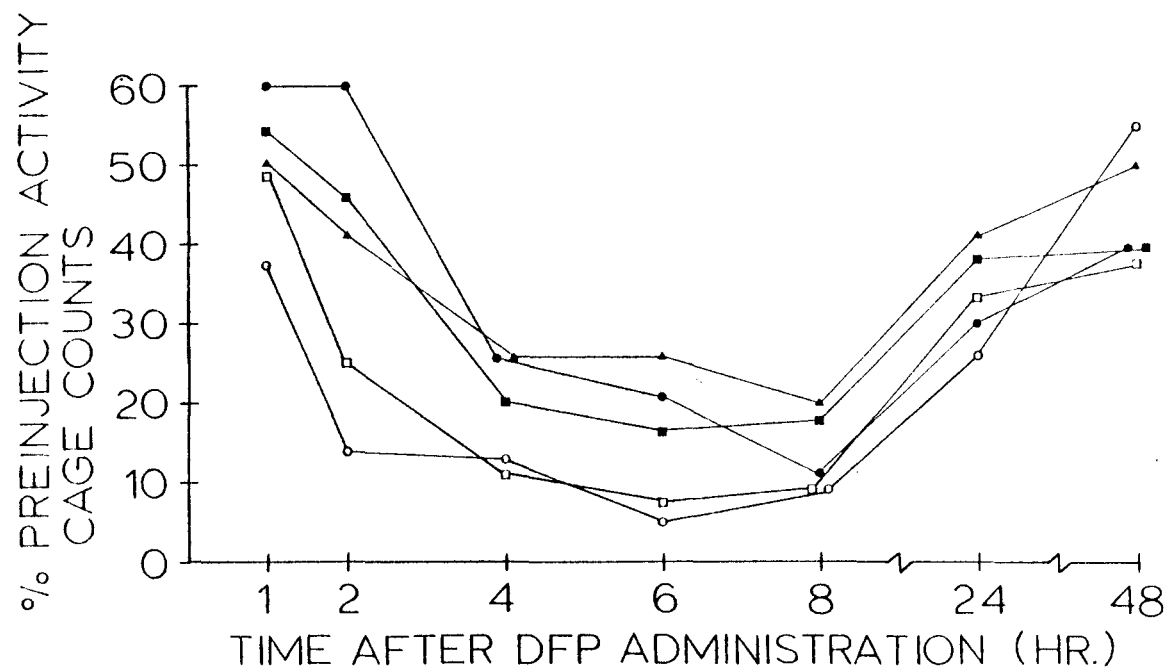
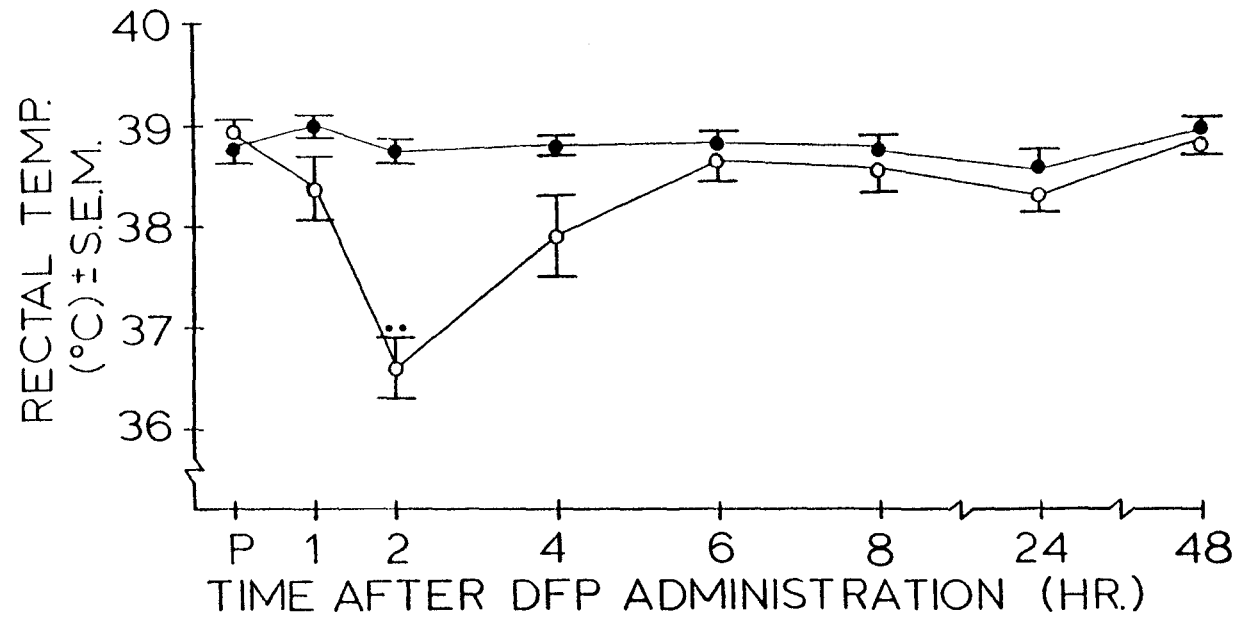


FIGURE 5

Rectal temperature measurements ( $^{\circ}\text{C}$ ) of rats subsequent to the administration of vehicle (closed circles) and DFP (1.0 mg/kg; open circles). Each point represents the mean  $\pm$  S.E.M. of eight animals. Rectal temperature measurements were compared to preinjection (P on abscissa) values for each animal (paired Student's t Test).

..p<0.01.

Fig. 5



#### 4.4 Brain Region Serotonin (5-HT) Levels of Rats

##### 4.4.1 Effect of DFP

Increased levels and turnover of acetylcholine (ACh; see section 1.2.3) and/or serotonin (5-HT; see section 1.3.2) in the central nervous system (CNS) have been associated with antinociception and analgesia. Since DFP markedly increased brain 5-HT levels when administered to rabbits (Barnes et al., 1975), it was important to ascertain whether DFP exerted a similar effect in the rat.

The effect of DFP on 5-HT levels in three rat brain regions was investigated one hour after the administration of DFP. As shown in Table 5, DFP (1.5 mg/kg) increased 5-HT levels in the medulla, meso-diencephalon, and telencephalon when compared to 5-HT levels of animals administered the vehicle, peanut oil (analysis of variance, Newman-Keuls test; Table 5). These increases amounted to 217, 132, and 131 per cent in the medullary, meso-diencephalic, and telencephalic brain regions respectively, with respect to animals administered the vehicle (Table 5). 5-HT levels of animals administered peanut oil, saline, or saline and peanut oil did not differ from values obtained in control, non-injected animals in any case (analysis of variance, Newman-Keuls test; Table 5).

##### 4.4.2 Effect of PCPA

To ascertain the significance of the rise in 5-HT with respect to the antinociception produced by DFP, PCPA, a tryptophan hydroxylase

TABLE 5

Serotonin (5-HT) levels expressed as ng 5-HT/gm tissue  $\pm$  S.D. in three rat brain regions (medulla, meso-diencephalon and telencephalon) subsequent to the administration of DFP (1.5 mg/kg), PCPA<sup>1</sup>, or PCPA<sup>2</sup> (see section 3.2.2), PCPA<sup>1</sup>-DFP (1.5 mg/kg), PCPA<sup>2</sup>-DFP (1.5 mg/kg) as well as vehicles. n = number of brain regions measured. In all cases, 5-HT levels in animals administered the various drug/s were compared to 5-HT levels obtained in animals administered the appropriate vehicle/s (analysis of variance, Newman-Keuls test).

\*\*p<0.01.

TABLE 5

EFFECT OF DFP, PCPA, AND PCPA-DFP ON RAT BRAIN REGION SEROTONIN (5-HT) LEVELSBRAIN REGION 5-HT LEVELS (ng 5-HT/gm TISSUE)  $\pm$  S.D.

<u>Group</u>	<u>(n)</u>	<u>Medulla</u>	<u>(n)</u>	<u>Mesodiencephalon</u>	<u>(n)</u>	<u>Telencephalon</u>
Control (non-injected)	12	270 $\pm$ 21	11	548 $\pm$ 26	11	339 $\pm$ 26
DFP (1.5 mg/kg)	7	589 $\pm$ 26**	7	724 $\pm$ 43**	8	444 $\pm$ 30**
Peanut oil	6	272 $\pm$ 11	8	518 $\pm$ 24	8	350 $\pm$ 21
PCPA <sup>1</sup>	6	202 $\pm$ 20**	5	196 $\pm$ 17**	6	237 $\pm$ 13**
PCPA <sup>2</sup>	8	143 $\pm$ 11**	8	159 $\pm$ 25**	8	196 $\pm$ 13**
Saline	6	259 $\pm$ 23	8	517 $\pm$ 44	8	379 $\pm$ 19
PCPA <sup>1</sup> -DFP (1.5 mg/kg)	6	213 $\pm$ 19**	7	234 $\pm$ 26**	9	282 $\pm$ 29
PCPA <sup>2</sup> -DFP (1.5 mg/kg)	6	173 $\pm$ 10**	6	187 $\pm$ 16**	6	227 $\pm$ 18**
Saline-peanut oil	7	279 $\pm$ 19	8	526 $\pm$ 30	8	348 $\pm$ 33

\*\*p&lt;0.01

inhibitor, was employed and its action on the DFP mediated changes in 5-HT levels, antinociception, and exploration and locomotion were evaluated.

First, the effect of PCPA on brain region 5-HT levels was studied; two dose regimens, PCPA<sup>1</sup> and PCPA<sup>2</sup>, were employed (see section 3.2.2); in either case, PCPA reduced 5-HT levels in all three brain regions; particularly PCPA<sup>2</sup> reduced 5-HT levels by 52, 70, and 48 per cent in the medullary, meso-diencephalic, and telencephalic brain regions, respectively, when compared to saline injected animals (analysis of variance, Newman-Keuls test; Table 5).

#### 4.4.3 Effect of PCPA-DFP

The effect of PCPA pretreatment regimens, PCPA<sup>1</sup> and PCPA<sup>2</sup>, on the DFP-induced increase in 5-HT levels was studied. Administered to PCPA<sup>1</sup> and PCPA<sup>2</sup> pretreated animals, DFP (1.5 mg/kg) did not affect the diminution in 5-HT levels produced by either PCPA<sup>1</sup> or PCPA<sup>2</sup> in any brain region studied (analysis of variance, Newman-Keuls test; Table 5). Brain 5-HT levels in PCPA<sup>1</sup>-DFP (1.5 mg/kg) and PCPA<sup>2</sup>-DFP (1.5 mg/kg) treated animals were still significantly reduced when compared to animals administered saline and peanut oil except for the effect of PCPA<sup>1</sup>-DFP (1.5 mg/kg) on the telencephalic 5-HT levels (analysis of variance, Newman-Keuls test; Table 5).

## 4.5 Effect of PCPA on DFP-induced Antinociception in Rats

### 4.5.1 Hot Plate Test

Since pretreatment with PCPA antagonized the DFP-induced increase in brain region 5-HT levels in rats, it was important to determine whether PCPA pretreatments would affect the antinociceptive state produced by DFP; the hot plate and tail flick tests were employed for this purpose.

Table 6 demonstrates the effect of two PCPA pretreatment regimens (PCPA<sup>1</sup> and PCPA<sup>2</sup>; see section 3.2.2) on the DFP-induced antinociceptive state of rats as measured by the hot plate test. Employed alone, PCPA<sup>1</sup> and PCPA<sup>2</sup>, produced hypernociception (Table 6). However, PCPA pretreatments did not antagonize the antinociceptive state produced by DFP (1.5 mg/kg; Table 6). Administration of the vehicles was ineffective (Table 6).

### 4.5.2 Tail Flick Test

PCPA<sup>2</sup> (see section 3.2.2) produced a hypernociceptive state when administered alone to rats as measured by the tail flick test (Table 7). However, PCPA<sup>2</sup> pretreatment did not affect the antinociceptive state produced by DFP (1.5 mg/kg; Table 7). Vehicles were ineffective in the tail flick test (Table 7).



TABLE 6

Hot plate response times (seconds  $\pm$  S.E.M.) of rats subsequent to the administration of vehicles, DFP (1.5 mg/kg), PCPA<sup>1</sup> or PCPA<sup>2</sup> (see section 3.2.2), as well as the combination PCPA<sup>1</sup>- or PCPA<sup>2</sup>-DFP (1.5 mg/kg). n = number of animals employed in each group. Post-injection responses were compared to preinjection responses for each animal (paired Student's t Test). \*p<0.05, \*\*p<0.01, and \*\*\*p<0.001.

TABLE 6

EFFECT OF PCPA ON DFP-INDUCED ANTINOCICEPTION:HOT PLATE TEST

<u>Group</u>	<u>(n)</u>	<u>Hot Plate</u>	
		<u>Response Times (Sec) <math>\pm</math> S.E.M.</u>	
		<u>Preinjection</u>	<u>Postinjection</u>
Control (non-injected)	10	8.1 $\pm$ 0.4	8.3 $\pm$ 0.4
Saline-peanut oil	10	6.9 $\pm$ 0.6	7.3 $\pm$ 0.4
DFP (1.5 mg/kg)	7	8.6 $\pm$ 0.9	15.3 $\pm$ 1.0**
PCPA <sup>1</sup>	12	7.1 $\pm$ 0.4	5.3 $\pm$ 0.4*
PCPA <sup>2</sup>	8	9.2 $\pm$ 1.1	5.4 $\pm$ 0.4**
PCPA <sup>1</sup> -DFP (1.5 mg/kg)	7	7.2 $\pm$ 0.7	21.2 $\pm$ 1.3***
PCPA <sup>2</sup> -DFP (1.5 mg/kg)	7	7.5 $\pm$ 0.5	21.4 $\pm$ 2.1***

\*p<0.05, \*\*p<0.01 and \*\*\*p<0.001

TABLE 7

Tail flick response times (seconds  $\pm$  S.E.M.) of rats subsequent to the administration of vehicles, DFP (1.5 mg/kg), PCPA<sup>2</sup> (see section 3.2.2), and PCPA<sup>2</sup>-DFP (1.5 mg/kg). n = number of animals employed in each group. Postinjection responses were compared to preinjection responses for each animal (paired Student's t Test). \*\*\*p<0.001.

TABLE 7

EFFECT OF PCPA ON DFP-INDUCED ANTINOCICEPTION:TAIL FLICK TEST

<u>Group</u>	<u>(n)</u>	<u>Tail Flick</u>	
		<u>Response Times (Sec) <math>\pm</math> S.E.M.</u>	
		<u>Preinjection</u>	<u>Postinjection</u>
Control (non-injected)	8	17.5 $\pm$ 0.5	18.1 $\pm$ 0.6
Saline-peanut oil	9	17.3 $\pm$ 0.5	16.9 $\pm$ 0.5
DFP (1.5 mg/kg)	8	17.2 $\pm$ 0.5	23.6 $\pm$ 0.6***
PCPA <sup>2</sup>	9	17.0 $\pm$ 0.5	10.7 $\pm$ 0.5***
PCPA <sup>2</sup> -DFP (1.5 mg/kg)	8	17.6 $\pm$ 0.7	25.8 $\pm$ 0.5***

\*\*\*p&lt;0.001

## 4.6 Effect of PCPA on DFP-induced Immobilization in Rats

### 4.6.1 Exploratory Activity

The effects of PCPA and PCPA-DFP combinations on exploratory and motor activity were evaluated to test whether or not the lack of effect of PCPA on DFP-induced antinociception was related to a PCPA action on the mobility of the rats. As shown previously (see section 4.2.1), DFP (1.5 mg/kg) decreased exploratory activity (Table 8). Administered alone, PCPA<sup>2</sup>, did not affect exploratory activity (Table 8). In addition, pretreatment with PCPA<sup>2</sup> did not affect the diminution in exploration produced by DFP (1.5 mg/kg; Table 8). Administration of the vehicles was ineffective (Table 8).

### 4.6.2 Motor Activity

As demonstrated earlier (see section 4.2.2), DFP (1.5 mg/kg) decreased motor activity; the diminution amounted to 61% when compared to animals administered the vehicle (Table 8). PCPA, administered by itself, significantly increased motor activity; yet, pretreatment with PCPA<sup>2</sup> did not affect the diminution in locomotion produced by DFP (1.5 mg/kg; Table 8). The vehicles did not affect motor activity (Table 8).

TABLE 8

Exploratory and motor activity levels (activity cage counts  $\pm$  S.E.M.) of rats subsequent to the administration of vehicles, DFP (1.5 mg/kg), PCPA<sup>2</sup> (see section 3.2.2), and PCPA<sup>2</sup>-DFP (1.5 mg/kg).

n = number of animals employed in each group.

Exploratory and motor activity levels were compared to levels obtained from animals administered the vehicles (analysis of variance, Newman-Keuls test).

\*\*p<0.01.

TABLE 8

EFFECT OF PCPA ON THE ATTENUATED EXPLORATORY AND  
MOTOR ACTIVITY LEVELS OF RATS PRODUCED BY DFP

<u>Group</u>	<u>(n)</u>	<u>Activity Cage Counts <math>\pm</math> S.E.M.</u>	
		<u>Exploratory</u>	<u>Motor</u>
Control (non-injected)	8	473 $\pm$ 18	521 $\pm$ 28
Saline-peanut oil	9	493 $\pm$ 24	543 $\pm$ 30
DFP (1.5 mg/kg)	8	257 $\pm$ 13**	211 $\pm$ 12**
PCPA <sup>2</sup>	9	372 $\pm$ 40	820 $\pm$ 21**
PCPA <sup>2</sup> -DFP (1.5 mg/kg)	8	177 $\pm$ 17**	183 $\pm$ 10**

\*\*p<0.01

#### 4.7 Pharmacological Comparison of DFP- and Morphine-induced Behavioral Effects in Rats

##### 4.7.1 Antinociception

In an attempt to ascertain whether DFP-induced antinociception is related to a narcotic sensitive and/or endogenous opioid system, the following experiments were performed.

The antinociceptive actions of DFP and morphine were evaluated pharmacologically; the hot plate test was employed for this analysis (Table 9). DFP (1.5 mg/kg) and morphine (3.0 mg/kg) produced equivalent levels of antinociception (Table 9). Atropine sulfate (4.0 mg/kg) antagonized the antinociceptive action of DFP but did not affect morphine-induced antinociception (Table 9). However, naloxone (5.0 mg/kg) antagonized both morphine- and DFP-induced antinociceptive states (Table 9). Atropine sulfate (4.0 mg/kg) and naloxone (5.0 mg/kg) were ineffective when administered alone. The vehicles were ineffective (Table 9).

##### 4.7.2 Exploratory Activity

The effect of DFP and morphine on exploration and locomotion was investigated pharmacologically to determine whether the antinociceptive state produced by these two compounds could be attributed to a drug effect on animal mobilization.

DFP (1.5 mg/kg) and morphine (3.0 mg/kg) reduced exploratory



TABLE 9

Hot plate response times (seconds  $\pm$  S.E.M.) of rats subsequent to the administrations of vehicles, DFP (1.5 mg/kg) and morphine (3.0 mg/kg) as well as DFP (1.5 mg/kg) or morphine (3.0 mg/kg) in combination with either atropine sulfate (4.0 mg/kg) or naloxone (5.0 mg/kg).  $n$  = the number of animals employed in each group. Postinjection responses were compared to preinjection responses for each animal (paired Student's  $t$  Test). \* $p < 0.05$  and \*\*\* $p < 0.001$ .

TABLE 9

PHARMACOLOGICAL COMPARISON OF DFP- AND MORPHINE-INDUCED ANTINOCICEPTIVE STATES: HOT PLATE TEST

<u>Group</u>	<u>(n)</u>	<u>Hot Plate</u>	
		<u>Response Times (Sec) <math>\pm</math> S.E.M.</u>	
		<u>Preinjection</u>	<u>Postinjection</u>
Control (non-injected)	12	8.2 $\pm$ 0.3	8.3 $\pm$ 0.4
Peanut oil-saline	10	8.6 $\pm$ 0.4	8.8 $\pm$ 0.4
DFP (1.5 mg/kg)	10	8.2 $\pm$ 0.3	16.9 $\pm$ 0.8***
DFP (1.5 mg/kg)-atropine sulfate (4.0 mg/kg)	8	8.4 $\pm$ 0.4	8.9 $\pm$ 0.5
DFP (1.5 mg/kg)-naloxone (5.0 mg/kg)	8	7.8 $\pm$ 0.4	8.5 $\pm$ 0.5
Morphine (3.0 mg/kg)	10	9.1 $\pm$ 0.3	17.3 $\pm$ 0.5***
Morphine (3.0 mg/kg)-atropine sulfate (4.0 mg/kg)	9	8.6 $\pm$ 0.5	19.4 $\pm$ 0.7***
Morphine (3.0 mg/kg)-naloxone (5.0 mg/kg)	8	8.9 $\pm$ 0.6	7.0 $\pm$ 0.4*

\* $p < 0.05$  and \*\*\* $p < 0.001$

activity levels (Table 10). The reduction in exploration by morphine or DFP was not affected by atropine sulfate (4.0 mg/kg) or naloxone (5.0 mg/kg), nor was the affect of DFP (1.5 mg/kg) affected by atropine methyl nitrate(4.0 mg/kg; Table 10). Administered alone, atropine sulfate(4.0 mg/kg) decreased exploratory activity; the vehicles were ineffective (Table 10). Exploratory activity levels were compared to levels obtained from animals administered the vehicles (analysis of variance, Newman-Keuls test; Table 10).

#### 4.7.3 Motor Activity

Employed at equi-antinociceptive doses, DFP (1.5 mg/kg) decreased, whereas morphine (3.0 mg/kg) increased motor activity (Table 10). Atropine sulfate (4.0 mg/kg), atropine methyl nitrate (4.0 mg/kg), and naloxone (5.0 mg/kg) did not affect the attenuated motor activity produced by DFP (1.5 mg/kg; Table 10). However, atropine sulfate (4.0 mg/kg) did antagonize the increase in motor activity produced by morphine (3.0 mg/kg); naloxone (5.0 mg/kg) not only antagonized but reversed the motor response induced by morphine (3.0 mg/kg; analysis of variance, Newman-Keuls test; Table 10). Administered alone, atropine sulfate (4.0 mg/kg) reduced motor activity; the vehicles were ineffective (Table 10). Motor activity levels were compared to levels obtained from animals which received the vehicles (analysis of variance, Newman-Keuls test; Table 10).

TABLE 10

Exploratory and motor activity levels (activity cage counts  $\pm$  S.E.M.) of rats subsequent to the administration of vehicles, atropine sulfate (4.0 mg/kg), DFP (1.5 mg/kg), and morphine (3.0 mg/kg) as well as DFP (1.5 mg/kg), or morphine (3.0 mg/kg) in combination with either atropine sulfate (4.0 mg/kg) or naloxone (5.0 mg/kg). n = number of animals employed in each group. Exploratory and motor activity levels were compared to levels obtained from animals administered the vehicle (analysis of variance, Newman-Keuls test). \*\*p<0.01.

TABLE 10

PHARMACOLOGICAL COMPARISON OF THE EFFECTS OF DFP AND MORPHINE ON EXPLORATORY AND MOTOR ACTIVITYLEVELS OF RATS

<u>Group</u>	<u>(n)</u>	<u>Activity Cage Counts <math>\pm</math> S.E.M.</u>	
		<u>Exploratory</u>	<u>Motor Activity</u>
Control (non-injected)	12	483 $\pm$ 17	524 $\pm$ 12
Peanut oil-saline	10	501 $\pm$ 21	511 $\pm$ 15
DFP (1.5 mg/kg)	10	248 $\pm$ 18**	236 $\pm$ 10**
DFP (1.5 mg/kg)-atropine sulfate (4.0 mg/kg)	8	311 $\pm$ 13**	249 $\pm$ 15**
DFP (1.5 mg/kg)-atropine methyl nitrate (4.0 mg/kg)	8	232 $\pm$ 19**	248 $\pm$ 18**
DFP (1.5 mg/kg)-naloxone (5.0 mg/kg)	8	198 $\pm$ 10**	196 $\pm$ 11**
Morphine (3.0 mg/kg)	10	324 $\pm$ 10**	791 $\pm$ 21**
Morphine (3.0 mg/kg)-atropine sulfate (4.0 mg/kg)	9	297 $\pm$ 15**	557 $\pm$ 32
Morphine (3.0 mg/kg)-naloxone (5.0 mg/kg)	8	293 $\pm$ 12**	158 $\pm$ 23**
Atropine sulfate (4.0 mg/kg)	6	325 $\pm$ 27**	390 $\pm$ 19**

\*\*p&lt;0.01

## 4.8 Effect of Naloxone on DFP-induced Responses in Rats

### 4.8.1 Antinociception

It was particularly noteworthy that naloxone, a pure narcotic antagonist, antagonized DFP-induced antinociception (see section 4.7.1). To further study this effect, the effect of naloxone (0.05, 0.5, and 5.0 mg/kg) on DFP-induced antinociception in rats was studied 1 to 48 hours after the single administration of DFP (1.0 mg/kg) utilizing the hot plate test. At the dose of 0.05 mg/kg, naloxone did not affect DFP-induced antinociception; it was effective 1 hour after the administration of DFP in antagonizing the DFP-induced antinociceptive state at doses of 0.5 and 5.0 mg/kg (Fig. 6). In fact, administered in a dose of 5.0 mg/kg, naloxone produced hypernociception 1 hour after the DFP administration (Fig. 6). Vehicles were ineffective (Fig. 6).

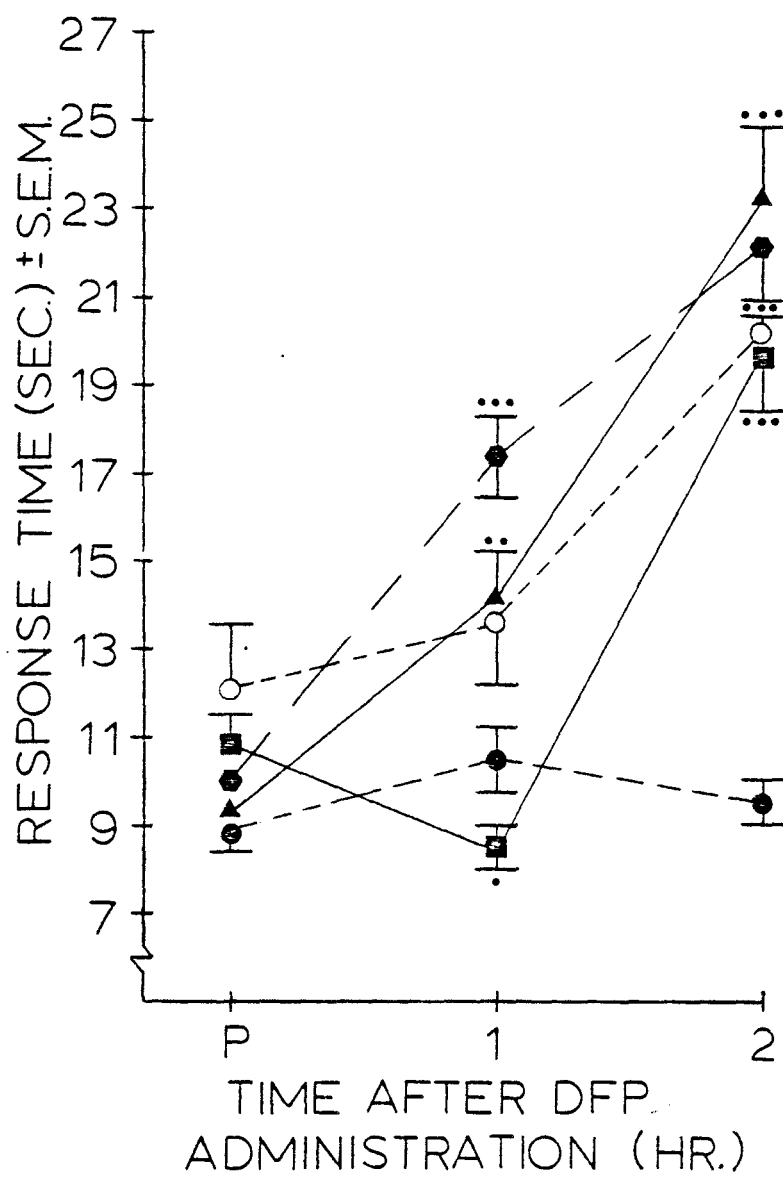
### 4.8.2 Exploratory Activity

As demonstrated earlier (see section 4.7.2), DFP (1.5 mg/kg) decreased exploration 1, 2, and 48 hours after the administration of DFP (Fig. 4). Naloxone in doses of 0.05, 0.5, and 5.0 mg/kg did not affect the attenuated exploratory activity produced by DFP (1.5 mg/kg). Figure 7A demonstrates exploratory activity levels 1, 2, and 4 hours after the DFP injection for animals administered the vehicles, DFP (1.5 mg/kg), and the combination DFP (1.5 mg/kg) with naloxone (5.0 mg/kg).

FIGURE 6

Hot plate response times (seconds  $\pm$  S.E.M.) of rats subsequent to the administrations of vehicles (closed circles); DFP (1.0 mg/kg) with naloxone (0.05 mg/kg-closed triangles; 0.5 mg/kg-open circles; 5.0 mg/kg-closed squares); and DFP (1.0 mg/kg with saline-closed hexagons). Each point represents the mean  $\pm$  S.E.M. of eight animals. Hot plate responses were compared to preinjection (P on abscissa) responses for each animal (paired Student's t Test).  $.p < 0.05$ ,  $..p < 0.01$ , and  $...p < 0.001$ .

Fig. 6





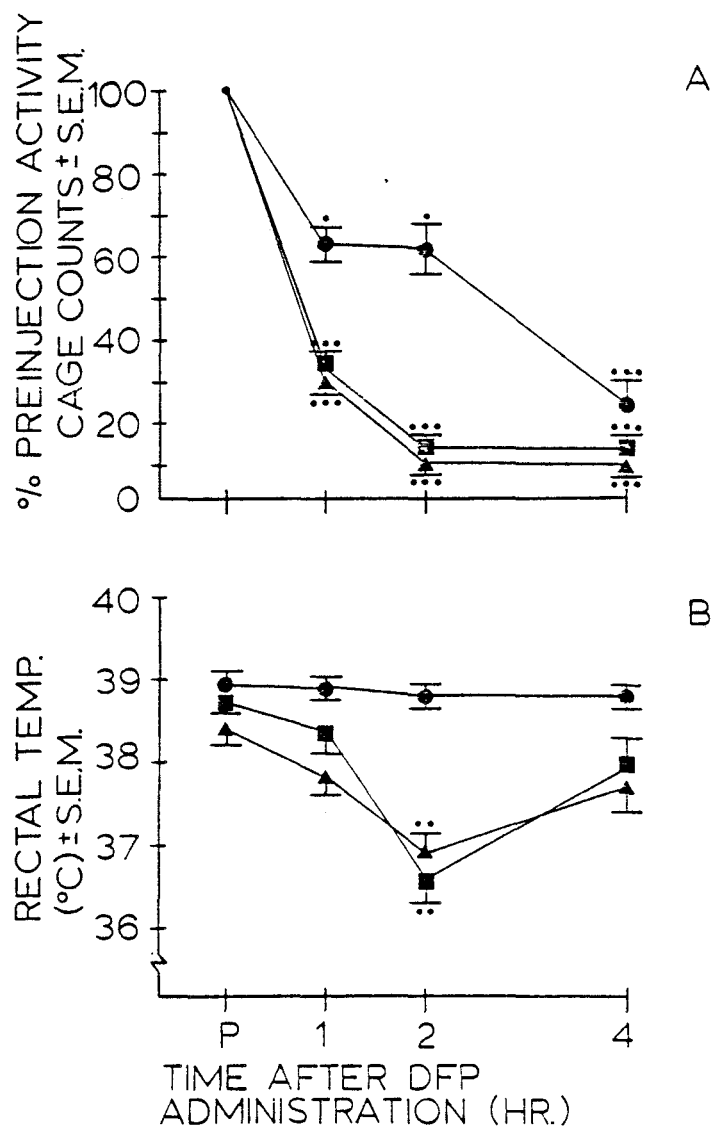
## FIGURE 7A

Exploratory activity levels of rats expressed as percent preinjection activity cage counts  $\pm$  S.E.M. subsequent to the administration of vehicles (circles), DFP (1.5 mg/kg) with saline (squares), and DFP (1.5 mg/kg)-naloxone (5.0 mg/kg) combination (triangles). Each point represents the mean  $\pm$  S.E.M. of five animals. Exploratory activity levels were compared to activity levels obtained from animals administered the vehicles (analysis of variance, Newman-Keuls test).  $..p<0.01$ .

## 7B

Rectal temperature measurements ( $^{\circ}\text{C}$ ) of rats subsequent to the administration of vehicles (circles), DFP (1.0 mg/kg) with saline (squares), and DFP (1.0 mg/kg)-naloxone (5.0 mg/kg) combination (triangles). Each point represents the mean  $\pm$  S.E.M. of eight animals. Rectal temperature measurements were compared to preinjection (P on abscissa) measurements for each animal (paired Student's t Test).  $..p<0.01$ .

Fig. 7



#### 4.8.3 Hypothermia

As demonstrated earlier (see section 4.3), DFP (1.0 mg/kg) produced hypothermia 2 hours after its administration (Fig. 7B). Naloxone (5.0 mg/kg) did not affect the hypothermic state produced by DFP (1.0 mg/kg) 2 hours after DFP (Fig. 7B). Administered alone, naloxone (5.0 mg/kg) did not affect rectal temperatures; the vehicles were ineffective (Fig. 7B).

Thus, naloxone reduced the antinociceptive state produced by DFP (see section 4.8.1) but did not affect the attenuated exploration (see section 4.8.2) or hypothermia (see section 4.8.3) produced by DFP.

#### 4.9 Effect of Stereoisomers of Narcotic Antagonists on DFP-induced Antinociception in Rats

Since non-specific actions of naloxone could contribute to its inhibition of DFP-induced antinociception, it was important to determine if the antagonism was stereospecific.

MR 2266, MR 2267, GPA 1843, GPA 1847, d-cyclazocine, and l-cyclazocine were administered individually 30 minutes after the administration of DFP (1.0 mg/kg) or morphine (3.0 mg/kg). MR 2266 (1.0 mg/kg) and GPA 1843 (5.0 mg/kg), the (-) isomers and active narcotic antagonists, reduced both morphine and DFP-induced antinociceptive states (Table 11). MR 2267 (1.0 mg/kg) and GPA 1847 (5.0 mg/kg), the corresponding (+) isomers, did not affect the antinociceptive states produced

TABLE 11

Hot plate response times (seconds  $\pm$  S.E.M.) of rats subsequent to the administrations of DFP (1.0 mg/kg) or morphine (3.0 mg/kg) followed 30 minutes later by either the vehicle, MR 2266 (1.0 mg/kg), MR 2267 (1.0 mg/kg), GPA 1843 (5.0 mg/kg), GPA 1847 (5.0 mg/kg), d-cyclazocine (0.64 mg/kg), or l-cyclazocine (0,64 mg/kg). n = number of animals employed in each group. Post-injection responses were compared to preinjection responses for each animal (paired Student's t Test). \*p<0.05 and \*\*p<0.01.

TABLE 11

EFFECT OF STEREOISOMERS OF NARCOTIC ANTAGONISTS ON  
MORPHINE AND DFP-INDUCED ANTINOCICEPTIVE STATES: HOT PLATE TEST

<u>Group</u>	<u>(n)</u>	<u>Hot Plate</u> <u>Response Times (Sec) <math>\pm</math> S.E.M.</u>	
		<u>Preinjection</u>	<u>Postinjection</u>
Morphine-saline	17	9.3 $\pm$ 1.2	18.6 $\pm$ 2.4**
Morphine-MR 2266 (-)	12	9.1 $\pm$ 0.9	9.8 $\pm$ 1.6
Morphine-MR 2267 (+)	12	9.0 $\pm$ 0.9	18.3 $\pm$ 2.9**
Morphine-GPA 1843 (-)	12	9.7 $\pm$ 0.7	12.2 $\pm$ 1.1
Morphine-GPA 1847 (+)	12	9.4 $\pm$ 0.8	16.5 $\pm$ 2.2**
Morphine-l,cyclazocine (-)	5	11.4 $\pm$ 1.0	13.6 $\pm$ 0.7
Morphine-d,cyclazocine (+)	5	11.1 $\pm$ 3.2	18.2 $\pm$ 3.2*
DFP-saline	13	8.8 $\pm$ 1.2	18.3 $\pm$ 2.7**
DFP-MR 2266 (-)	8	6.7 $\pm$ 0.7	7.4 $\pm$ 1.2
DFP-MR 2267 (+)	8	8.7 $\pm$ 1.4	12.2 $\pm$ 2.8*
DFP-GPA 1843 (-)	8	10.8 $\pm$ 1.9	12.2 $\pm$ 1.5
DFP-GPA 1847 (+)	8	8.8 $\pm$ 1.9	15.4 $\pm$ 3.1*
DFP-l,cyclazocine (-)	5	9.7 $\pm$ 0.7	16.7 $\pm$ 1.7*
DFP-d,cyclazocine (+)	5	11.2 $\pm$ 1.3	10.6 $\pm$ 1.4

duced by either morphine or DFP (Table 11). The vehicle was ineffective (Table 11).

On the other hand, the relationship between the antagonist action against DFP- and morphine-induced antinociception and the stereoisomers of cyclazocine, a partial narcotic agonist, was reversed; d-cyclazocine (0.64 mg/kg) antagonized the antinociceptive action of DFP but did not affect morphine-induced antinociception; l-cyclazocine (0.64 mg/kg) reduced morphine-induced antinociception but was ineffective against DFP-induced antinociception (Table 11).

#### 4.10 Effect of Tolerance to Morphine on DFP- and Pilocarpine-induced Antinociception in Rats

Cross tolerance studies were performed to discern a possible common neural mechanism mediating antinociception produced by morphine, pilocarpine, and DFP. As demonstrated earlier (see sections 4.1.1 and 4.7.1) DFP (1.0 mg/kg), pilocarpine (2.5 mg/kg), and morphine (3.0 mg/kg) produced antinociception as measured by the hot plate test in rats (Pretolerance; Table 12). Morphine-induced antinociception was completely attenuated in animals rendered tolerant to morphine as measured on day 6 (tolerance level A; Table 12). However, DFP- and pilocarpine-induced antinociception was maintained in animals rendered tolerant to morphine on day 6 (tolerance level A); in addition, DFP-induced antinociception was maintained on day 9 (tolerance level B; Table 12). Thus, the antinociceptive actions of DFP and pilocarpine did not exhibit cross tolerance to the antinociceptive

TABLE 12

Hot plate response times (seconds  $\pm$  S.E.M.) of rats subsequent to administrations of DFP (1.0 mg/kg), pilocarpine (2.5 mg/kg), or morphine (3.0 mg/kg). Hot plate measurements were made both prior to the development of tolerance (Pretolerance) as well as after the development of tolerance to morphine at different levels (Tolerance levels A and B; see section 3.2.3). n = number of animals employed in each group. All responses were compared to Preinjection, baseline, responses for each animal (paired Student's t Test). \*p<0.05 and \*\*p<0.01.

TABLE 12

EFFECT OF TOLERANCE TO MORPHINE ON DFP- AND PILOCARPINE-INDUCEDANTINOCICEPTION: HOT PLATE TEST

<u>Administration</u>	<u>Hot Plate</u>		
	<u>Response Times (sec) <math>\pm</math> S.E.M.</u>		
	<u>Morphine</u> (n=8)	<u>DFP</u> (n=8)	<u>Pilocarpine</u> (n=8)
Preinjection	11.0 $\pm$ 0.6	11.0 $\pm$ 1.1	7.0 $\pm$ 0.7
Pretolerance	22.7 $\pm$ 1.1**	21.2 $\pm$ 1.4**	12.3 $\pm$ 2.2*
Tolerance Level A	11.4 $\pm$ 1.0	17.6 $\pm$ 1.7*	13.3 $\pm$ 2.6*
Tolerance Level B	-	17.3 $\pm$ 2.0*	-



actions of morphine. Tolerant animals exhibited no altered responses when administered the vehicle alone. Non-tolerant animals demonstrated antinociceptive responses to morphine (3.0 mg/kg) and DFP (1.0 mg/kg) on days 1, 6, and 9; responses to pilocarpine (2.5 mg/kg) were not measured on these days.

#### 4.11 Effect of DFP on Rat Brain Enkephalin-like Material Levels

The stereospecificity of the narcotic antagonist action on the antinociceptive state produced by DFP (see section 4.9) suggests that DFP-induced antinociception is mediated via opiate receptors, possibly by the release of endogenous opioids. Therefore, the effects of DFP on whole brain enkephalin-like-material levels in the rat were studied 1 hour after the administration of DFP.

Sixty-eight rat brains were assayed for enkephalin-like-material content utilizing the mouse vas deferens bioassay technique as described in section 3.6. Unfortunately, only 7 rat brain samples were found to be acceptable according to the criteria of the bioassay procedure (see section 3.6.5.3). Enkephalin-like-material content of whole rat brains, expressed as ng leucine-enkephalin equivalents/gm brain tissue, was determined to be 229 ng leucine-enkephalin equivalents/gm brain tissue in animals which received DFP (1.0 mg/kg; n=2), and 252 ng leucine-enkephalin equivalents/gm brain tissue in animals which received peanut oil (n=5). These levels were not significantly different (analysis of variance, Newman-Keuls test).

The following reasons explain why, for the most part, the remaining brain samples (n=61) failed to meet acceptable bioassay criteria and therefore could not be included in the results: (i) per cent recovery of the internal standards (see section 3.6.4) often was less than 90% and occasionally even as low as 20% in some experiments, (ii) the rat brain sample increased the resting tone of the longitudinal muscle of the mouse vas deferens making it impossible to accurately measure the depression of the electrically-evoked twitch height (Fig. 8A), (iii) the rat brain sample inconsistently depressed the electrically-evoked twitch height of the longitudinal muscle of the mouse vas deferens, that is, the depressed twitch height returned to normal prior to washout time; this suggests that substances other than enkephalin-like-material were responsible for the twitch height depression (Fig. 8B), and (iv) the depression of the electrically-evoked twitch height of the longitudinal muscle of the mouse vas deferens produced by the rat brain sample could not be completely (that is by 100%) reversed by the addition of naloxone (900 nM bath concentration); this indicates that additional not enkephalin-like-materials may be responsible for the twitch height depression (Fig. 8C).

Overall, the experiments designed to determine the effect of DFP on rat brain enkephalin-like-material levels were incomplete and inconclusive due to the lack of reliable, extraction and bioassay techniques.

## FIGURE 8A

Inhibition of the electrically-evoked contraction of the longitudinal muscle of the mouse vas deferens by a rat brain sample extract (S). Notice the increased resting tone of the vas deferens produced by S when compared to the normal resting tone following the administration of standard leucine-enkephalin (see Figure 2). W = washout time.

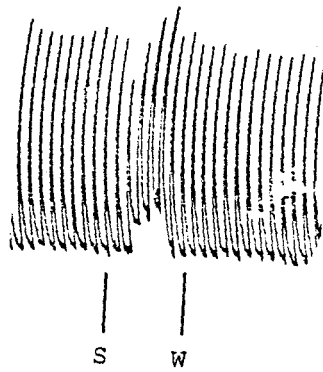
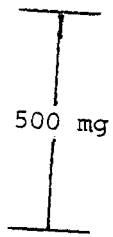
## 8B

Inhibition of the electrically-evoked contractions of the longitudinal muscle of the mouse vas deferens by a rat brain sample extract (S). Notice the non-constant depression of twitch height produced by S when compared to the constant twitch height depression produced by standard leucine enkephalin (see Figure 2). W = washout time.

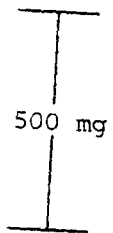
## 8C

Inhibition of the electrically-evoked contractions of the longitudinal muscle of the mouse vas deferens by a rat brain sample extract (S) and subsequent reversal by the addition of naloxone (N; 900 nM bath concentration) prior to washout (W). Notice the incomplete, less than 100%, reversibility by N of the depressed twitch height produced by S when compared to the complete reversal of the twitch height depression produced by standard leucine-enkephalin (see Figure 2).

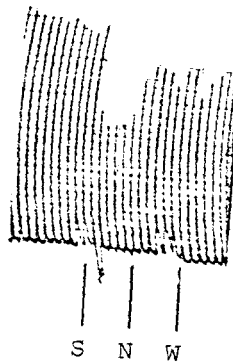
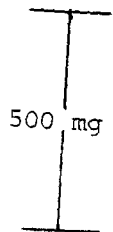
Fig. 8



(A)



(B)



(C)

## 5. DISCUSSION

### 5.1 General

#### 5.1.1 DFP, Mechanism of Action

Anticholinesterase (anti-ChE) agents constitute one of the few classes of drugs for which a mechanism of action has been defined in terms of inhibition of a specific enzyme. Until World War II, the enzyme cholinesterase (ChE) was of pure academic interest; however, during the war, intense research programs arose in the field of organophosphorus anti-ChE's because their toxicities suggested their use as potential chemical warfare agents. Investigations in England produced a series of organophosphorus anti-ChE's, one of which was diisopropyl phosphofluoridate (DFP; Holmstedt, 1959; Karczmar, 1970).

Cholinesterases (ChE's) contain at least one anionic and one esteratic site in their active centers; the most likely carrier of the negative charge in the anionic site is glutamic acid while serine and histidine are the basic groups of the esteratic site (Karczmar, 1970).

True cholinesterase (AChE) which hydrolyzes acetylcholine at a higher rate than butyrylcholine is the enzyme present in the brain, spinal cord, striated muscles, smooth muscles of the bronchioles, urinary bladder, salivary glands, and erythrocytes of several but not all vertebrates (Koelle, 1951). Pseudocholinesterase (BuChE) which hydrolyzes butyrylcholine at a higher rate than acetylcholine is found

in the serum of most vertebrates, carotid body, hepatic cells, and muscularis mucosa of intestinal cells (Koelle, 1951). DFP produces a 50% inhibition of BuChE at low concentrations ( $10^{-8}$ M) while AChE is 50% inhibited by higher concentrations of DFP ( $10^{-6}$ M); thus, DFP preferentially inhibits BuChE (Aldridge, 1953). The doses of DFP used in this dissertation, 0.1 to 1.5 mg/kg may be extrapolated in terms of equidistribution in the tissues as corresponding to  $5.4 \times 10^{-7}$  to  $8.2 \times 10^{-6}$ M. Thus, it would be expected that DFP in doses of 0.5, 1.0, and 1.5 mg/kg inhibit at least 50% of AChE; less than 50% inhibition of AChE would be expected from the administration of 0.1 mg/kg DFP. Actually, as an extremely lipid soluble compound (Usdin, 1970), DFP may be expected to be distributed preferentially in the nerve tissue.

Anti-ChE's may be classified as reversible or irreversible based upon the ease with which the inhibitory action can be reversed. Reversible inhibition has been divided into three groups: (i) competitive (ii) noncompetitive and (iii) uncompetitive. Competitive inhibition occurs with anti-ChE's which are structurally similar to the normal substrate; with these compounds,  $K_m$  increases and  $V_{max}$  is unaltered. Noncompetitive inhibitors bind with some site on the ChE molecule distinct from the active site; in this case,  $V_{max}$  decreases and  $K_m$  remains unaltered. Uncompetitive inhibition occurs when the inhibition binds to the enzyme substance complex; in this case,  $K_m$  and  $V_{max}$  both decrease. Oximes, inorganic compounds, and bis and mono quaternary compounds are examples of reversible inhibitors (Karczmar, 1970).

The inhibitory action of irreversible inhibitors is not easily reversed and the amount of inhibition increases as the amount of inhibitor present increases. Irreversible inhibition occurs in a two step process (i) a reversible formation of enzyme-inhibitor complex and (ii) an irreversible phosphorylation (carbamylation) of the enzyme; the degree of inhibition depends upon the rate constant of inhibition and is directly proportional to the amount of inhibitor present. Organophosphorus compounds such as DFP, carbamates, and organosulfonates are irreversible inhibitors (Usdin, 1970).

DFP contains an electron deficient phosphorus atom by virtue of the attached highly electronegative fluorine atom. This phosphorus atom makes an electrophilic attack on the electron rich oxygen atom of the serine residue yielding diisopropylphosphoserine, a fluorine atom, and a proton. Thus, DFP enters the active site of serine containing enzymes including ChE's, phosphorylates the serine residue, and inactivates the enzyme (Jansz et al., 1959; Koshland, 1963).

The major mechanism of action of DFP, as well as the other ChE inhibitors, is actually due to the accumulation of ACh within the body as a result of continued release from nerve terminals and failure to be subsequently hydrolyzed by active ChE (Usdin, 1970). Thus, the pharmacological effects of DFP are for the most part predictable based upon a knowledge of those sites where ACh is released by and the corresponding effector organs of the chemical mediator.

Single incremental subcutaneous (s.c.) administrations of DFP

in rats produced (i) skeletal muscle twitches; (ii) increased salivation, retching, diarrhea, and micturition; and (iii) general flaccidity; death when it occurred was attributed to respiratory failure, caused in part by the neuromuscular paralysis, central depression, and increased bronchiole secretions. Lethal dose-50 (LD-50) established in our laboratories and elsewhere amounted 3.0 mg/kg (Horton et al., 1946); it depends on the source and the batch of DFP.

In humans, the systemic administration of DFP produced the following: (i) twitching and generalized weakness of skeletal muscles; (ii) bronchoconstriction and increased secretions; (iii) increased sweating, salivation, lacrimation, and micturition; (iv) gastrointestinal disorders; and (v) slight bradycardia (Grob, 1963). Central nervous system (CNS) manifestations included giddiness, tension excessive dreaming, insomnia, nightmares, headache, electroencephalographic (EEG) alterations, drowsiness, confusion, and generalized depression of respiratory and circulatory centers (Grob, 1963).

Finally, a delayed, demyelinating neuropathy characterized by an onset of sensory symptoms and motor weakness has been reported 8 to 14 days after exposure to DFP and other organophosphorus compounds; the mechanism responsible for this effect remains to be determined (Grob, 1963).

#### 5.1.2 DFP, Investigational Compound

DFP was chosen as the investigational compound for these studies for several reasons. First, cholinergic-induced antinociception



arises from stimulation of central cholinergic receptors (see section 1.2.3). Drugs administered systemically must therefore be able to cross the blood brain barrier (BBB) to produce antinociception. DFP, being highly lipophilic readily penetrates the BBB.

Second, the irreversible inhibition of ChE produced by DFP allows the various biochemical and behavioral experimental procedures to be performed at times distant from injection or one another, reducing the injection frequency and handling stress effects (see section 3.3.1).

Third, both the serotonergic (see section 1.3) and cholinergic (see section 1.2) systems have been implicated in antinociception. That the serotonergic system is coupled with the cholinergic system is suggested by the fact that pilocarpine, arecoline, oxotremorine, DFP, and physostigmine increase serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels in rats indicating an increased 5-HT turnover (Reid, 1970; Haubrich and Reid, 1972; Barnes et al., 1978). In addition, DFP increased 5-HT levels to a greater extent than did the other compounds. This research was initiated to separate the cholinergic from the serotonergic effects with respect to cholinergic-induced antinociception. DFP, which produced the greatest effect on 5-HT levels, appeared to be best suited for this purpose.

Finally, the ability to produce antinociception when administered to laboratory animals had not yet been demonstrated for DFP. In fact, DFP failed to produce antinociception in previous studies; however, experimental procedures involved differed from those employed pres-

ently (Saxena, 1958; Cox and Tha, 1972). Saxena (1958) utilized noxious pressure applied to the rats tail; the hot plate and tail flick tests were used here. Cox and Tha (1972) used mice; rats were studied in this laboratory.

### 5.1.3 Methods of Evaluating Antinociception

Antinociception can be measured by a variety of laboratory testing procedures in several laboratory animals. For instance, electrical stimulation of the tooth pulp in the dog, cat, and rabbit has been useful in determining antinociceptive activity since the tooth pulp contains nociceptors (see section 1.1.4.1).

Pressure or noxious mechanical stimulation applied to the tail of the rat or mouse has been used as a means of evaluating antinociceptive activity. This method, originally described by Haffner (1929), utilized an artery clip applied to the base of the tail; the latency of biting at the clip was quantitated.

The intraperitoneal (i.p.) administration of a number of compounds such as ACh, acetic acid, and phenylquinone produce a characteristic response in mice called writhing. The ability of compounds to inhibit this response has also been used to assess antinociceptive activity.

The classical method of experimentation employs noxious heat stimuli to evaluate the antinociceptive activity of drugs. The original method of D'Amour and Smith (1941) involved focusing a beam of

of light through a prism onto a rats tail; the latency of the characteristic tail flick was quantitated.

The reaction of a mouse or rat placed on a hot plate, noxious heat source, has also been used extensively to evaluate antinociceptive activity. The original paper of Woolfe and MacDonald (1944) described the responses observed when mice were placed on a hot plate; included here were sitting up, blowing on the front paws so as to cool them, licking of the hind paws, and eventually jumping off of the hot plate surface.

Overall, the simplest and yet effective method is the use of the hot plate. By placing the animal, which has received the test drugs, on the hot plate surface and recording the time to response it is possible to determine the relative potency, onset, intensity, and duration of antinociceptive activity. Thus, the hot plate test was used in these experiments.

The application of heat to the rats tail is another method which may prove useful in the evaluation of antinociception since the characteristic tail flick response represents a spinally mediated reflex which appears to be independent of motor behavior; thus, this test also was used here.

The choice of animals and procedures was dependent also on the following considerations; (i) the hot plate and tail flick tests are easily performed requiring no additional surgical procedures as in the tooth pulp stimulation experiments and no additional injections as in

the writhing tests, (ii) the choice of antinociception testing procedures limited the choice of laboratory animals to be used to the rat or mouse. The decisive factor was the ability to extract sufficient quantities of 5-HT and enkephalin-like-material for quantitative analysis of the brain.

## 5.2 DFP-Induced Antinociception

DFP produced antinociception when administered to rats whether measured by the hot plate or tail flick test (Tables 1 and 2). The antinociceptive state produced by DFP followed a dose dependent relationship as determined by the hot plate procedure (Table 1). Antinociception produced by DFP was long lasting, up to 6 hours duration in the hot plate test, which is consistent with the irreversible effect of DFP on ChE (Fig. 3).

When one considers that besides being bound to ChE's, DFP is also bound irreversibly to non-ChE proteins and proteolipids and as it is readily hydrolyzed enzymatically (Mazur, 1946), DFP is a particularly potent antinociceptive agent, as at the dose of 1.5 mg/kg it increased by 214 and 132% the animals response times in the hot plate and tail flick tests, respectively (Tables 1 and 2).

It is noteworthy that even at low doses (0.1 mg/kg), DFP produced a significant antinociceptive response (Table 1). While DFP is considered as acting primarily via the inhibition of ChE with resultant ACh accumulation, it appears that anti-ChE's may have actions independent of ChE inhibition (Van Meter et al., 1978). Therefore, DFP may

produce antinociception by actions independent of ChE inhibition such as affecting ionic conductances or rendering the cholinergic receptor more reactive to ACh (Van Meter et al., 1978).

Cholinergic-induced antinociception and analgesia result from actions involving central cholinceptive sites of the muscarinic type (see section 1.2.3). Similarly, DFP-induced antinociception appears to result from central actions involving muscarinic receptors since atropine sulfate antagonized while atropine methyl nitrate was ineffective in blocking this response (Table 2).

Specific central sites involved in mediating the antinociceptive effects of DFP cannot be determined from these studies. However, additional experiments which would provide evidence for specific sites of action could be performed as follows.

First, spinal and supraspinal structures may be associated with DFP-induced antinociception. Assessment of tail flick response in animals in which the spinal cord has been transected (T6-T8) may provide evidence for a principal spinal or supraspinal site of action. Second, previous investigations suggest that certain structures such as the septum, mesencephalic reticular formation, and medial thalamic nuclei may be involved in mediating cholinergic-induced antinociception (see section 1.2.3). Localized injections of DFP with the concomitant assessment of nociceptive responses may therefore provide additional evidence for an anatomical substrate for the particular DFP effect.

Third, cholinergic-induced antinociception may be indirectly mediated through the involvement of other neurotransmitter systems in the CNS as suggested earlier (see section 1.2.4). Various pharmacological manipulations can be used to ascertain which neurotransmitter system or systems as well as which tracts or areas are involved. In fact, this principle was utilized to determine the involvement of the serotonergic system in DFP-induced antinociception (see section 5.5). Similar studies could be designed to determine the involvement of other neurotransmitter systems such as the noradrenergic and dopaminergic systems.

Another problem is concerned with the clinical application of the data. Although DFP is too toxic to be employed in clinical studies, other anti-ChE's such as physostigmine can be utilized to gain information concerning a possible site of action and mechanism of cholinergic-induced antinociception. The assessment of pain in a clinical setting may provide evidence to suggest which component of the pain experience, sensory-discriminative, motivational-affective, or cognitive-evaluative, is affected by the drug. Since specific brain mechanisms are thought to be related to specific anatomical structures data obtained in the clinics may provide evidence for not only which brain process but also which anatomical loci are involved in cholinergic-induced antinociception (Melzack and Casey, 1968; Melzack, 1975).

Recently, Sitaram et al. (1977) have demonstrated that physostigmine produced analgesia in normal human volunteers as measured by

(i) the subjects verbal response and (ii) specific cortical average evoked electroencephalographic responses (AER). Sitaram et al. (1977) suggest that physostigmine affects the motivational-affective and cognitive-evaluative psychological components of the pain experience via its cortical arousal effect. This dissertation provides the basis for continued clinical investigations concerned with the involvement of the cholinergic system in the pain phenomenon. In particular, the effect of physostigmine on narcotic-induced analgesia will be studied (appendix 1). See section 5.6.3 and appendix 1 for a complete discussion of the appended clinical investigation protocol.

Finally, one problem concerning the present data should be emphasized. Hot plate responses of animals administered either 0.1 mg/kg or 0.5 mg/kg of DFP or the drug vehicle were attenuated 6 to 8 hours post injection (Fig. 3). Two explanations concerning this effect may be presented as follows. The attenuation of hot plate responses could be attributed to a learning/conditioning effect; repeated exposure to a task involving a negative consequence (hot plate surface) decreases the time associated in the task. Alternatively, the animals were exhibiting a diurnal variation in responsiveness to noxious input. Significant diurnal variations have been demonstrated for hot plate response times in mice (Frederickson et al., 1977). Thus, the animals administered the vehicle or DFP in low doses (0.1 and 0.5 mg/kg) may have been exhibiting a normal diurnal variation in responsiveness; employed in doses of 1.0 and 1.5 mg/kg DFP was so potent as not to allow this expression of a diurnal rhythm (Fig. 3).

### 5.3 DFP-Induced Immobilization

The first question which arises is whether the antinociceptive state produced by DFP is due to or dependent on, the attenuation of spontaneous locomotor activity; this is particularly pertinent in the case of the hot plate test since the behavioral responses which are measured in this test involve locomotor movements (see section 3.3.2).

The animals exploratory activity level, that is its response to a novel environment, may be a pertinent correlate of antinociception as measured by the hot plate test since both measurements were made immediately after placing the animal in this novel environment.

DFP reduced exploratory activity levels; neither atropine sulfate nor atropine methyl nitrate affected the attenuation of exploration produced by DFP (Tables 4 and 10). In fact, atropine sulfate also reduced exploration when administered alone; this may account for its inability to restore exploratory activity attenuated by DFP (Table 10). Yet, DFP-induced antinociception was antagonized by atropine sulfate; atropine sulfate, alone, failed to alter hot plate responses (Table 3). Thus, the antinociceptive state produced by DFP is not related to the attenuated exploration produced by DFP.

An apparent discrepancy arises concerning the effect of anticholinergic agents on exploration (see section 1.2.4), as many investigators reported that atropine augments exploratory and/or motor activity. However, this discrepancy can be explained by examining



the operational definitions employed in each case. Komisaruk (1970) defined exploratory activity in terms of sniffing behaviors; Abeelen et al. (1972) regarded rearing, leaning, and sniffing as exploratory acts; presently, exploration was defined in terms of gross locomotor movements during early phase of exposure to novel surroundings (see section 3.3.4.2). Clearly, while all studies measured behaviors in a novel environment, exploration, these studies do not measure identical behaviors.

Additional evidence that the antinociception and attenuation of exploration produced by DFP are independent phenomena is as follows: (i) peak attenuation of exploration did not coincide with peak antinociceptive responses at any time tested 1 to 48 hours after the administration of any dose of DFP, (ii) at 0.1 mg/kg, DFP produced antinociception but did not affect exploratory activity, and (iii) naloxone antagonized DFP-induced antinociception but did not affect DFP-induced attenuation of exploration (Tables 9 and 10). A complete discussion of the effect of naloxone on DFP-induced behavioral responses is provided in section 5.6.

Motor activity levels which represent the animals ability to make spontaneous locomotor movements also were studied as a potential correlate to antinociception. It should be pointed out that motor activity was measured 15 minutes after the animals had been placed in the activity cages while hot plate responses were measured immediately after placing the animal on the hot plate surface(see section 3.3.4.2).

DFP reduced in a dose dependent manner motor activity in rats; the diminution in motor activity produced by DFP was not affected by atropine sulfate, atropine methyl nitrate, or naloxone (Tables 4 and 10). However, employed by itself, atropine sulfate reduced motor activity; this may account for its inability to restore motor activity attenuated by DFP (Table 10). On the other hand, DFP-induced antinociception was antagonized by atropine sulfate and naloxone; atropine methyl nitrate was ineffective (Table 9). Thus, DFP-induced antinociception was sensitive to the actions of both atropine sulfate and naloxone whilst DFP-induced attenuation of motor activity was not affected by either atropine sulfate or naloxone. Therefore, the antinociception and attenuation of motor activity produced by DFP do not appear to be related.

Finally, it must be emphasized that DFP produced antinociception in the tail flick test as well; this test appears to be less dependent on changes in motor behavior than the hot plate test. Since antinociception and the attenuation of locomotion appear to be independent, further experiments to separate these two phenomena need not be proposed.

#### 5.4 DFP-Induced Hypothermia

Since the hot plate and tail flick tests involve temperature sensitive measurements and as the systemic administration of DFP is known to produce hypothermia (Meeter and Wolthius, 1968), the hypothermic state produced by DFP may interfere with the evaluation of

## DFP-induced antinociception.

In agreement with the earlier data of Meeter and Wolthius (1968), DFP produced a pronounced hypothermia (Fig. 5). Here, DFP-induced antinociception and hypothermia were seemingly independent effects: (i) DFP (1.0 mg/kg) produced antinociception which lasted up to 6 hours post administration (Fig. 3) while hypothermia was obtained only at 2 hours post injection (Fig. 5) and, (ii) naloxone antagonized the DFP-induced antinociceptive state (Table 8) but did not affect hypothermia produced by DFP (Fig. 7). A complete description of the effect of naloxone on DFP-induced behavioral responses is given in section 5.6.

Liu and Fong (1972) demonstrated that hypothermia produced an alteration in the antinociceptive response to intra-arterial injections of ACh in cats; mild hypothermia, a 2 to 3° decrease in rectal temperature below normal, produced a hypernociceptive state; a further reduction in rectal temperature, 5 to 6° below normal rectal temperature, produced antinociception. Based upon the study of Liu and Fong (1972), the hypothermia produced by DFP in the present experiments, a 2.4° decrease in rectal temperature, would be expected to produce hypernociception and thus antagonize the antinociceptive state produced by DFP. In fact, DFP produced both a pronounced level of antinociception and hypothermia in the rat and thus it appears that DFP-induced antinociception and hypothermia are unrelated.

## 5.5 DFP-Induced antinociception and the Serotonergic System

### 5.5.1 Neurochemistry

The next question which arises concerns the neurochemical mechanisms involved in DFP-induced antinociception. The antinociceptive state and ACh levels and/or turnover rates may be correlated for some but not all centrally acting cholinomimetics, anticholinesterases, narcotic agonists and antagonists, and various CNS depressants (see section 1.2.3). Thus, the antinociceptive state produced by those compounds may not be directly attributable to actions via the central cholinergic system. The multitransmitter character of the CNS as well as the effects which cholinergic agents produce on systems other than the cholinergic system suggest the possibility that DFP-induced antinociception may be attributed to a DFP action mediated by non-cholinergic systems (Karczmar, 1975).

First, the interplay between the cholinergic and serotonergic systems may be involved. For a long time now the serotonergic system has been implicated in antinociception; increased activity of brain and spinal cord serotonin neurons such as that produced by electrical stimulation, precursor loading, uptake inhibitors, and receptor agonists is associated with antinociception and enhanced antinociceptive potency of drugs such as morphine; decreased activity in those neurons induced by electrical and chemical lesioning as well as precursor restricted diets is associated with hypernociception and diminished antinociceptive drug potency (see section 1.3).

That the serotonergic system may be coupled with the cholinergic system is suggested by the fact that oxotremorine and physostigmine (Reid, 1970), pilocarpine and arecoline (Haubrich and Reid, 1972), and DFP (Barnes et al., 1974, 1975) increase 5-HT and 5-HIAA levels; these data indicate that DFP increased 5-HT turnover, and indeed appropriate measurements showed that this is so (Barnes et al., unpublished).

It was therefore important to determine if DFP had a similar effect in rats. As shown in Table 5, 5-HT levels were markedly increased in the rat medulla, meso-diencephalon, and telencephalon following a single administration of DFP. DFP increase in 5-HT levels and turnover as well as the antinociceptive state produced by DFP are mediated through activation of central muscarinic receptors since atropine sulfate antagonized whereas atropine methyl nitrate did not affect these two effects of DFP (Barnes et al., 1978; Koehn and Karczmar, 1978).

It may be speculated that the resultant accumulation of ACh due to DFP inhibition of ChE depolarizes serotonergic neurons thus increasing 5-HT levels and turnover. On the other hand, the increase in 5-HT levels and turnover elicited by DFP might be due to a direct stimulatory action of DFP upon 5-HT release. The present experiments do not demonstrate which explanation is correct. Finally, DFP may increase 5-HIAA brain levels by affecting transport systems responsible for 5-HIAA elimination.

Since the rise in 5-HT levels and turnover produced by DFP could underlie DFP-induced antinociception, it was important to separate these two phenomena produced by DFP. One method by which this could be accomplished is by manipulating the serotonergic system followed by DFP administration, measurements of nociception and of 5-HT levels. Para-chlorophenylalanine (PCPA) which inhibits the synthesis of 5-HT by inhibiting tryptophan hydroxylation was employed for this purpose.

The use of PCPA as an inhibitor of 5-HT biosynthesis was first reported by Koe and Weissman (1966). The mechanism of action of PCPA on the 5-HT biosynthetic process appears to involve three separate parts. These various mechanisms must be considered in experiments in which PCPA is used to evaluate biochemical mechanism underlying behavioral and pharmacological phenomenon.

First, a reversible inhibition of tryptophan hydroxylase (TH) activity lasting 4 hours with peak effect at 1 to 2 hours post injection was observed following the systemic administration of PCPA; this inhibition of enzyme activity was attributed to 1) competition of PCPA with substrate for entry into the nerve ending as well as 2) the competitive inhibition of the enzyme for the substrate (Knapp and Mandell, 1972). A second, irreversible inhibition of TH was observed 2 days after the PCPA injection; this inhibition has been attributed to incorporation of PCPA into TH during new protein synthesis in the nerve cell body (Gal et al., 1970). Thus, pretreatment of the animal two days as well as 2 hours prior to experimentation yields a model

system in which the three mechanisms involved in the inhibition of 5-HT synthesis by TH are maximally inhibited. In fact, this was the basis of the injection schedules employed presently (PCPA<sup>1</sup> and PCPA<sup>2</sup>; see section 3.2.2).

Finally, the doses of PCPA utilized in these experiments were selected because these doses specifically inhibited TH activity without affecting the activity of other enzymes such as tyrosine hydroxylase, aromatic amino acid decarboxylase, or monoamine oxidase all of which affect dopaminergic or noradrenergic neurotransmitter systems (Koe and Weissman, 1966).

Serotonin (5-HT) levels were measured in rat medulla, meso-diencephalon, and telencephalon subsequent to the administration of PCPA<sup>1</sup> or PCPA<sup>2</sup> (see section 3.2.2) to monitor the inhibitory action of PCPA on 5-HT biosynthesis. As shown in Table 5, PCPA decreased 5-HT levels in all three rat brain regions, particularly following the two daily 300 mg/kg doses followed on the third day by a 100 mg/kg dose (PCPA<sup>2</sup>). It should be pointed out that the depletion of rat brain 5-HT was not as great as that originally described by Koe and Weissman (1966). However, the experimental procedures in these two studies differed in the following ways. First, Koe and Weissman (1966) sacrificed the rats by an intravenous (i.v.) administration of pentobarbital; decapitation was used in the present studies (see section 3.5.1). Second, spectrophotofluorometric methods for quantitation of 5-HT differed; Koe and Weissman (1966) measured 5-HT directly whereas, here, 5-HT

was converted to a substituted indole compound which is more fluorescent than 5-HT itself (see section 3.5.5).

Subsequently, the effect of DFP on rat brain region 5-HT levels in animals pretreated with either PCPA<sup>1</sup> or PCPA<sup>2</sup> was determined (see section 3.2.2). DFP did not affect attenuation of 5-HT levels by PCPA pretreatment dosage regimens in any of the three rat brain regions studied; that is, PCPA pretreatment prevented the DFP-induced augmentation of 5-HT levels (Table 5). Thus, DFP-induced increase in rat brain 5-HT levels depends upon a functionally intact 5-HT biosynthetic system.

#### 5.5.2 Antinociception

Since the DFP augmentation of 5-HT levels in rat brain depends upon a functionally intact 5-HT biosynthetic system, it was important to determine whether the antinociceptive state produced by DFP also depends upon this system.

PCPA produced hypernociceptive responses in rats as measured by the hot plate test (Table 6). This effect of PCPA is consistent with earlier findings of Tenen (1967) and Messing et al. (1975) who demonstrated an increased sensitivity to electric footshock in rats as measured by the flinch-jump test.

The tail flick test was also employed to assess the nociceptive responses of rats following PCPA treatment. Here, the tail flick apparatus was adjusted so that the response latency of control animals



was long enough (15 seconds; see section 4.5.2) to allow monitoring of possible hypernociceptive responses. As a result, it would be shown that PCPA produced hypernociception in rats also as measured by the tail flick test (Table 7). Thus, just as in the case of previous reports (see section 1.3), decreased serotonergic neurotransmission such as that produced by PCPA correlates with the hypernociceptive state in rats.

As demonstrated earlier (see sections 4.1.1 and 4.1.2), DFP produced antinociception in rats as measured by the hot plate (Table 6) and tail flick tests (Table 7). If DFP-induced antinociception were dependent upon a functionally intact 5-HT system, the pretreatment with PCPA would be expected to antagonize the antinociceptive action of DFP. Alternatively, if DFP-induced antinociception were independent of the 5-HT system, PCPA pretreatment would not be expected to affect the DFP-induced antinociceptive state. Tables 6 and 7 demonstrate the effect of PCPA pretreatment on the DFP-induced antinociceptive state as measured by the hot plate and tail flick tests, respectively. The interesting finding was that PCPA pretreatment did not antagonize DFP-induced antinociception as evaluated by either test (Tables 6 and 7). Thus, DFP-induced antinociception and the increase in brain 5-HT levels produced by DFP appear to be independent effects. The augmentation of rat brain 5-HT levels is dependent whereas antinociception produced by DFP is independent of a functionally intact 5-HT biosynthetic system.

Generally, the antinociceptive effects of physostigmine were

affected by manipulation of the serotonergic system (see section 1.2.3.3). This discrepancy concerning the effect of PCPA pretreatment on cholinergic-induced antinociception (see section 1.2.3.3) may be due to differences in the mechanism of the cholinergic effects of oxotremorine, DFP and physostigmine. Thus, it should not be surprising that the antinociceptive states produced by these compounds may arise through different mechanisms. In fact, in the case of oxotremorine and physostigmine this was found to be so; oxotremorine-induced antinociception is potentiated by procedures which decrease brain catecholamine systems but is unaffected by procedures which change the brain 5-HT system; physostigmine-induced antinociception is affected by procedures which alter catecholamine and 5-HT systems (Pleuvry and Tobias, 1971). It should also be noted that with the exception of the present study, none of the other investigations measured 5-HT levels and/or turnover and thus were unable to correlate behavioral and neurochemical data. Finally, differences in technique and dosages must also be considered in this context.

#### 5.5.3 Immobilization

It has long been known that PCPA affects spontaneous locomotor activity in the rat (Tenen, 1967). Since the hot plate test involves locomotor sensitive measurements, the effects of PCPA and PCPA-DFP combinations were evaluated to determine whether the lack of effect of PCPA on DFP-induced antinociception was related to a PCPA action on the mobility of the rat.

Previous investigations have demonstrated that PCPA decreased exploration in rats (Marsden and Curzon, 1976), contrary to the present findings. However, it should be noted that Marsden and Curzon (1976) defined exploration in terms of open field activities such as the number of squares entered, number of rearings, number of 180° turns, grooming time, and number of fecal pellets; thus, their operational definition of exploration was different from the locomotor activity levels measured as exploration in the present experiments (see section 3.3.4.2).

As demonstrated earlier (see section 4.2.1), DFP reduced exploratory activity levels of rats (Table 8). PCPA, administered by itself, did not affect exploration nor was it capable of affecting the diminution of exploratory activity produced by DFP (Table 8). It would be expected that via reducing exploratory activity in rats PCPA could interfere with the assessment of the nociceptive response. However, since PCPA did not affect exploration nor did pretreatment with PCPA affect the diminution of exploration produced by DFP, the lack of effect of PCPA on DFP-induced antinociception was not related to an alteration of exploration produced by PCPA.

As demonstrated earlier (see section 4.2.2), DFP reduced motor activity levels of rats (Table 8). PCPA, administered by itself, increased motor activity (Table 8); this effect of PCPA on motor activity is consistent with the original findings of Tenen (1967). However, PCPA was unable to affect the DFP-induced decrease in motor activity (Table 8). If PCPA affected the antinociceptive state produced

by DFP by altering the animals motor activity levels, it would be expected that the PCPA-induced increase in motor activity would antagonize DFP-induced antinociception. However, PCPA pretreatment did not antagonize the antinociception produced by DFP (Table 8). Thus, the PCPA affect on motor activity was not related to the PCPA lack of effect on DFP-induced antinociception.

## 5.6 DFP-induced Antinociception and the Endogenous Opioid System

### 5.6.1 Antinociception, Pharmacological Analysis

It has long been known that anti-ChE's such as prostigmine (Slaughter and Munsell, 1940), physostigmine (Floodmark and Wrammer, 1940), and DFP (Bhargava and Way, 1972); cholinomimetic compounds such as pilocarpine (Saxena and Gupta, 1957); as well as ACh (Pedigo et al., 1975) itself, potentiate narcotic-induced antinociception and/or analgesia. Recent discoveries of the stereospecific opiate receptor (Goldstein et al., 1971) and several endogenous opioid-like peptides, enkephalins (Hughes et al., 1975a) and endorphins (Li and Chung, 1976; Guillemin et al., 1976) have advanced our working knowledge of pain mechanisms. Therefore, any hypothesized role for a neurotransmitter such as ACh in pain modulation must take into account possible interactions with endogenous opioid system. A pharmacological analysis of the antinociceptive actions of DFP and morphine was performed to investigate possible relationships between the cholinergic and endogenous opioid systems in the pain phenomenon.

As described earlier (see section 4.1.1), DFP produced antinociception in the rat as measured by the hot plate test (Table 9). As expected, morphine also produced antinociception in rats as measured by the hot plate test; this effect was antagonized by the narcotic antagonist, naloxone; atropine sulfate was ineffective (Table 9). The interesting finding of this study was the fact that naloxone antagonized DFP-induced antinociception (Table 9).

Naloxone has been reported to block the antinociceptive effects of other compounds which affect the cholinergic system such as physostigmine and oxotremorine (Harris et al., 1969) and ACh (Pedigo et al., 1975) as well as other pharmacologically- and physiologically-induced antinociceptive states such as those produced by acupuncture (Mayer et al., 1976), stimulation produced analgesia (SPA; Akil et al., 1976), and stress (Bodnar et al., 1977; Chesher and Chan, 1977). The fact that naloxone antagonized the antinociception arising from these various procedures suggests that antinociception may result from actions mediated through the opiate receptor, possibly via the release of endogenous opioids.

However, it must be pointed out that naloxone antagonism of antinociception is necessary but not sufficient evidence to implicate the involvement of the endogenous opioid system. Nonspecific actions of naloxone could interfere with the assessment of nociceptive responses. First, naloxone may act as a gamma amino butyric acid (GABA) antagonist. The iontophoretic application of naloxone antagonized GABA-

induced inhibition of firing in 21 out of 27 spontaneously active, olfactory tubercle neurons in the rat (Dingledine et al., 1978). Furthermore, applied iontophoretically, naloxone antagonized ACh, nicotine and morphine-induced excitation but did not affect morphine-induced inhibition of Renshaw cells in cats (Duggan et al., 1975). Thus, naloxone also may act as a cholinergic-nicotinic receptor antagonist. Alternative explanations describing the naloxone effect are also plausible. Finally, naloxone itself, has been shown in some cases to produce analgesia. Lasagna (1965) demonstrated a bidirectional effect of naloxone; low doses of naloxone, 1 to 2 mg, produced analgesia whereas higher doses, 5 to 8 mg, produced hyperalgesia as measured by verbal reports in patients with postoperative pain. Recently, naloxone has been shown to produce analgesia measured as changes in cortical evoked potentials following electrical stimulation, in pain-sensitive individuals; the same dose of naloxone produced hyperalgesia in pain-sensitive individuals (Buschsbaum et al., 1977).

Experiments which would help define the role of the endogenous opioids include the following. First, stereoisomers of narcotic antagonists other than naloxone may be used to determine the stereospecificity of the narcotic antagonism. Experiments with MR 1452/3 and MR 2266/7, the  $\alpha$  methyl and  $\alpha$  ethyl N furylmethyl analogues of  $\alpha$ -5,9-dialkyl-2'-hydroxy-6,7-benzomorphan, respectively and GPA 1843/7, the N-allyl analogue of  $\beta$ -9-methyl-5-phenyl-2'-hydroxy-6,7-benzomorphan, have confirmed the stereospecific effects of naloxone on the electrically evoked release of ACh from guinea pig ileum (Waterfield and

Kosterlitz, 1975). Jacob and Ramabadran (1978) also have confirmed stereospecific effects of MR 2266/7 on the morphine-induced antinociceptive state in mice utilizing the hot plate test. The effect of stereoisomers of narcotic antagonists on DFP-induced antinociception is discussed in section 5.6.2 below.

Next, the existence of cross tolerance with the antinociceptive action of morphine may be used to demonstrate the involvement of a common neural substrate. Both exogenous  $\beta$ -endorphin (Szekely et al., 1977) and methionine-enkephalin (Blasig and Herz, 1976) produce cross tolerance with morphine. In addition, tolerance to morphine reduced the effective level of antinociception produced by electrical stimulation (SPA) of periaqueductal gray areas (PAG; Mayer and Hayes, 1975) and stress in rats (Spiaggia et al., 1979); reciprocal tolerance was incomplete in each case (Mayer and Hayes, 1975; Spiaggia et al., 1979). Cross tolerance studies between DFP- and morphine-induced antinociception are described in section 5.6.3).

Direct release of endogenous opioid peptides by an appropriate stimulus constitutes further evidence for the involvement of the endogenous opioid system. Electrical stimulation (SPA) of the periaqueductal gray region (PAG), which produced analgesia in patients suffering from chronic intractable pain, was associated with increased cerebrospinal fluid (CSF) levels of enkephalin-like-material (Akil et al., 1978) and immunoreactive- $\beta$ -endorphin (Hosobuchi et al., 1979). Electro-acupuncture increased endorphin activity CSF levels in chronic

pain patients (Sjolund et al., 1977). The effect of DFP on the endogenous opioid system is discussed below in section 5.6.4.

Additional experiments that would clarify the mechanisms involved in DFP-induced antinociception may be suggested. Pomeranz et al. (1977) demonstrated that electro-acupuncture-induced antinociception could be blocked by hypophysectomy in mice. Li and Chung (1976) and Guillemin et al. (1976) have reported that morphine-like-pituitary-peptides, endorphins, are concentrated in the pituitary. If DFP-induced antinociception was mediated via the release of endorphins from pituitary sites, then hypophysectomy would be expected to block the antinociceptive action of DFP. Carboxypeptidase A and leucine aminopeptidase are known to enzymatically degrade enkephalins (Hughes et al., 1975a). In addition, the enkephalins are rapidly degraded by enzymes found in rat cerebral tissues (Dupont et al., 1977). If DFP-induced antinociception were mediated by the release of enkephalins, agents which inhibit the degradative enzymes should potentiate this response. For example, bacitracin is known to inhibit the enzymatic breakdown of enkephalins in rat brain tissue extracts (Simantov et al., 1976c). However, the specificity and potential side effects associated with any enzyme inhibitor which would otherwise interfere with nociception measurements, must be considered.

#### 5.6.2 Stereospecificity

Since non-specific actions of naloxone could contribute to its inhibition of DFP-induced antinociception (see section 5.6.1), it was



important to determine whether the antagonism was stereospecific; synthetic antagonists of the benzomorphan series were employed for this purpose. MR 2266, MR 2267 and GPA 1847 possess relatively low agonists activity while GPA 1843 possesses no agonist activity as measured by their abilities to depress the electrically evoked contraction of the guinea-pig ileum (Waterfield and Kosterlitz, 1975). The (-) isomers of two narcotic antagonists, MR 2266 and GPA 1843, antagonized both morphine and DFP-induced antinociceptive responses whereas the corresponding (+) isomers, MR 2267 and GPA 1847, did not reduce either morphine or DFP-induced antinociceptive responses (Table 11). This stereospecificity of narcotic antagonist action suggests that DFP-induced antinociception is indeed mediated via opiate receptors. Jacob and Ramabadran (1978) have demonstrated similar effects of MR 2266 and MR 2267 on antinociception produced by arecoline and physostigmine.

Although Pert and Snyder (1973) could not find affinity of ACh, atropine, or carbamylcholine for stereospecific opiate receptors, the possibility remains that DFP may itself act directly on the opiate receptor; appropriate binding studies are required.

Stereoisomers of cyclazocine, a partial narcotic agonist, had a differential effect on the antinociceptive states produced by DFP and morphine; (-) cyclazocine antagonized whereas (+) cyclazocine did not affect morphine-induced antinociception; alternatively (+) cyclazocine antagonized DFP-induced antinociception while (-) cyclazocine

zocine was ineffective (Table 11). This effect has been reported previously employing stereoisomers of cyclazocine and pentazocine (Pedigo et al., 1975). The discrepancy between the action of narcotic antagonists and partial narcotic agonists on cholinergic-induced antinociception is particularly puzzling. The type of test employed or drug specificity does not explain this effect as different tests for measuring nociceptive responses and different compounds which affect the cholinergic system have produced similar results (Pedigo et al., 1975; Table 11). No plausible interpretation of this effect can be presented at present.

Finally, inconsistencies in the actions of the (+) isomers (MR 2267, GPA 1847, and d-cyclazocine, Table 11) suggest that different opiate receptors may mediate the antinociceptive actions of DFP and morphine. Additional investigations such as  $pA_2$  studies are required to clarify these effects.

### 5.6.3 Cross Tolerance Studies

Cross tolerance exists between the antinociceptive responses to morphine and exogenous opioid peptides (Blasig and Herz, 1976; Szekely et al., 1977). It would be expected that if DFP- and morphine-induced antinociception were mediated via a common neuronal substrate, cross tolerance to morphine and DFP would be observed (see section 5.6.1). However, cross tolerance did not develop between morphine-induced antinociception and the antinociceptive

state produced by DFP (Table 12). Cross tolerance studies between morphine and pilocarpine were performed to determine whether the lack of cross tolerance between morphine and DFP was specific for DFP or representative of cholinergic-induced antinociception. Pilocarpine was selected since it enhances cholinergic neurotransmission by different mechanisms than does DFP (see section 5.1.1); pilocarpine mimicks directly the action of ACh on cholinergic-muscarinic receptors. Again, cross tolerance failed to develop between the antinociceptive actions of morphine and pilocarpine (Table 12). A similar lack of cross tolerance between morphine and oxotremorine (Howes et al., 1969) and physostigmine (Pleuvry and Tobias, 1971) has been reported. Thus, it appears that morphine- and cholinergic-induced antinociception is not cross tolerant.

The possibility that the level of morphine tolerance was inadequate to demonstrate cross tolerance with DFP appears unlikely since DFP maintained an antinociceptive action even at increased levels of morphine tolerance (tolerance level B, Table 12).

Alternatively, the lack of cross tolerance suggests that multiple neural substrates may act via opiate receptors to produce

antinociception. The chronic administration of morphine while producing tolerance to exogenous opioid peptides (Blasig and Herz, 1976; Szekely et al., 1977) may not induce tolerance at those sites in the CNS at which a DFP- and pilocarpine-endogenous opioid interactions occur.

Distinct opiate receptor populations which exhibit selective high affinity binding for (D-ala, D-leu)-enkephalin and morphine have been identified in the rat CNS (Chang and Cuatrecasas, 1979). It has not been established whether morphine, exogenous opioid peptides, or endogenous opioid peptides normally interact with one or both opiate receptors. However, it may be that cholinergic compounds release endogenous enkephalins which then interact with one type of opiate receptor while morphine interacts with another opiate receptor. Whether cross tolerance occurs between these two opiate receptor populations remains to be established.

The basis for implementing the appended clinical investigation protocol (see appendix 1) may be reviewed in light of these present findings as to the lack of cross tolerance between morphine and DFP.

Anti-ChE's administered by themselves, are known to produce antinociception (Harris et al., 1968; Koehn and Karczmar, 1978) and analgesia (Sitaram et al., 1977); in addition, anti-ChE's potentiate narcotic-induced antinociception (Bhargava and Way, 1972; Ireson, 1970) and analgesia (Floodmark and Wrammer, 1940). The antinociceptive actions of anti-ChE's do not exhibit cross tolerance with the antinociceptive action of morphine (Pleuvry and Tobias, 1971; Koehn et al., 1979). The appended clinical investigation protocol (see appendix 1) was designed to determine whether the anti-ChE's agent, physostigmine, would produce analgesia in narcotic tolerant patients. It is hoped that this study will provide evidence for the existence of a cholinergic sensitive system which may be manipulated pharmacologically for the treatment of pain.

#### 5.6.4 Effect of DFP on the Endogenous Opioid System

The release of endogenous opioid peptides by an appropriate stimulus is required to establish the involvement of the endogenous opioid system in antinociception. If DFP-induced antinociception arises via actions involving the endogenous opioid system, DFP should release endogenous opioids. Potassium-induced depolarizations have been shown to release methionine- and leucine-enkephalin from isolated striatal slices (Henderson et al., 1978) as well as  $\beta$ -endorphin from pituitary and hypothalamic sites (Przewlocki et al., 1978; Osborne et al., 1978). The present experiments were designed to study the effect of DFP on enkephalin-like material release from rat brain. Experimental procedures for extraction (Hughes et al., 1977) and quantitation (Hughes et

al., 1975b) of enkephalin-like-material were chosen for this purpose (see section 3.6). In fact, the studies of the effects of DFP on enkephalin-like-material did not yield adequate data due to the lack of reliable extraction and bioassay techniques (see section 4.11).

Methodological requirements for release studies include the following. First, the extraction process should eliminate any contaminants which would otherwise interfere with the quantitation procedure. This, in fact, was the primary failure in the present experiments (see section 4.11). Second, the assay technique must be appropriately sensitive to measure experimentally-induced changes; here, sensitivities of 0.5 ng leucine-enkephalin equivalents are required, and this sensitivity did not obtain in our experiments. Enkephalin-like-material levels in rats were studied with and without a DFP injection in an attempt to establish proper methodology required for release studies as well as to demonstrate an effect of DFP on enkephalin-like-material content (see section 4.11). While only 7 out of 68 rat brain samples studied fulfilled acceptable criteria for bioassay (see section 3.6.5.3), no significant differences in enkephalin-like-material levels were observed between animals administered DFP or the drug vehicle. The failure of the remaining brain samples to meet acceptable criteria was due to the presence of a non-enkephalin-like-material contaminant (see section 4.11).

Several changes implemented to eliminate any impurities or contaminants can be outlined as follows: (i) the chromatography resin was recycled an additional 10 times with isopropyl alcohol and water

(see section 3.6.3.1), (ii) brain homogenates were centrifuged at between 5 and 120,000 G for between 10 to 60 minutes (see section 3.6.4), (iii) height of the resin bed was changed to 40, 80 and 120 mm (see section 3.6.3.2), (iv) eluate was put through columns to reelute enkephalin-like-material (see section 3.6.4), (v) eluate was taken to complete dryness by freeze evaporation techniques under vacuum with phosphorus pentoxide as drying agent whenever samples would not go to complete dryness in Rotorvapor Apparatus (see section 3.6.4), (vi) freeze dried samples were heated (see section 3.6.4), and (vii) brain samples were passed through Amberlite IRA-400 anion exchange resin prior to introduction onto Amberlite XAD-2 columns (see section 3.6.4). Invariably, these changes failed to improve the extraction process.

Although the experimental procedures utilized in these experiments failed to produce results, alternative methods are available. Radioimmunoassay techniques have been used to determine the enkephalin content of several rat brain regions (Yang et al., 1977; Kobayashi et al., 1978b). The sensitivity and specificity of the antisera employed in the radioimmunoassay must be taken into account for quantitative analysis of this type.

#### 5.6.5 Exploratory and Motor Activity

An established effect of DFP treatment is reduction of the animal's locomotion (see section 1.2.4). Since the effects of DFP and morphine on rat mobility and environmental response may interfere with the hot plate assessment of antinociception, exploratory and motor

activity effects of these drugs with and without their antagonists were studied.

As described earlier (see section 5.3), DFP reduced exploratory activity levels in rats; neither atropine sulfate, atropine methyl nitrate, nor naloxone affected the DFP reduction of exploration (Table 10). Morphine also reduced exploration in rats; naloxone and atropine sulfate did not alter this morphine effect (Table 10). However, the antinociceptive states produced by DFP and morphine do not depend upon the reduction of exploration produced by either compound since naloxone antagonized the antinociceptive state but failed to alter the attenuated exploration produced by these two compounds.

It must be pointed out that atropine sulfate, alone, reduced exploration; this may account for its lack of effect in reversing the DFP-induced reduction in exploratory activity. A similar effect of naloxone, alone, could account for its failure to reverse the reduction in exploration produced by morphine. Since the effect of naloxone on exploration was not studied in the present experiments, further investigations are required to clarify this point.

As described earlier (see section 5.3), DFP reduced motor activity levels of rats; atropine sulfate, atropine methyl nitrate, and naloxone did not affect the diminution in locomotion produced by DFP (Table 10). On the other hand, morphine increased locomotion; atropine sulfate prevented while naloxone reversed the morphine-induced increase in motor activity (Table 10).



Section 5.3 describes the effect of DFP on locomotion, indicating that DFP-induced antinociception and DFP-induced attenuation of locomotion are not related. In addition, the endogenous opioid system does not appear to participate in the DFP-induced depression of locomotion since naloxone failed to alter this DFP effect.

It is well recognized that morphine affects spontaneous locomotor activity in rats. Low doses of morphine (1.0 to 5.0 mg/kg) increase locomotion while higher doses of morphine (10 to 40 mg/kg) produced biphasic changes, initial depression followed by stimulation of locomotion (Domino et al., 1976). In the present experiments, morphine (3.0 mg/kg) increased locomotion (Table 10). The locomotor effect of morphine appears to be mediated via opiate receptors since naloxone antagonized this effect (Table 10). Naloxone not only antagonized but actually reversed the effect of morphine on locomotion (Table 10). However, since the effect of naloxone, alone, was not studied it is impossible to determine whether the action of naloxone on morphine depression of locomotion was due to the effect of the former on motor behavior or involved the endogenous opioid system. Other investigators failed to demonstrate an effect of naloxone on motor activity (Bhargava, 1978b; Amir et al., 1979).

It is interesting to note that atropine sulfate antagonized the increased locomotion produced by morphine thus implicating the cholinergic system in this particular morphine effect (Table 10). Recent studies have shown that the effect of morphine on locomotion and ACh synthesis in rats correlates for some but not all doses of morphine;

thus, the depressant and/or stimulant action of morphine on locomotion may involve neurotransmitter systems other than the cholinergic system (Vaskó and Domino, 1978).

Finally, the antinociceptive effects of morphine and DFP do not appear to be related to the effect of these drugs on motor activity since (i) employed at equiactive antinociceptive doses, morphine increased while DFP decreased locomotion, (ii) atropine sulfate and naloxone antagonized DFP-induced antinociception but failed to alter the attenuation of locomotion produced by DFP, (iii) atropine sulfate antagonized the augmented motor activity produced by morphine but failed to alter morphine-induced antinociception (Table 10).

#### 5.6.6 Effect of Naloxone on DFP-induced Responses

The effect of naloxone on antinociception, attenuated mobility, and hypothermia produced by DFP was studied 1 to 48 hours after the DFP injection to determine if the naloxone antagonism of DFP-induced antinociception was related to a naloxone effect on animal mobility or temperature.

As discussed earlier (see section 4.7.1), naloxone antagonized DFP-induced antinociception in a dose dependent manner (Fig. 6). Naloxone (5.0 mg/kg) not only antagonized DFP-induced antinociception but also produced hypernociception following the DFP administration (Fig. 6). Naloxone has been shown to produce hypernociception only under certain circumstances such as stress (Madden et al., 1977; Rossier et al., 1977a). Thus, the reversal of DFP-

induced antinociception may be attributed in part to a naloxone effect in a stressed animal. It should also be pointed out that naloxone (5.0 mg/kg) produced different effects on DFP-induced antinociception in different studies; naloxone (5.0 mg/kg) antagonized DFP-induced antinociception in earlier studies (see section 5.6.1).

That the duration of action of DFP was greater than that of naloxone correlates well with the irreversible nature of this anti-ChE agent (Fig. 6). In addition, the antinociceptive response induced by morphine similarity outlasts the antagonistic action of naloxone (Smits, 1976).

As described earlier (see section 4.8.2), naloxone failed to alter DFP-induced decrease in exploration 1 to 48 hours post DFP injection (Fig. 7A). Naloxone also failed to affect DFP-induced attenuation of motor activity (Table 10). Thus, the naloxone antagonism of DFP-induced antinociception is not related to a naloxone effect on DFP-induced immobilization.

As discussed earlier (see section 4.3), DFP produced hypothermia in rats. Naloxone did not affect the hypothermia produced by DFP nor did it alter rectal temperatures in naive animals (Fig. 7B). Since naloxone antagonized DFP-induced antinociception (Table 9) but failed to affect the hypothermia produced by DFP (Fig. 7B), the naloxone antagonism of DFP-induced antinociception is independent of a naloxone temperature effect.

### 5.7 Concluding Comments

Pain, by itself, is an extremely complex, ill-defined phenomenon (see section 1). All research investigations concerning the various aspects of the pain phenomenon entail experimental procedures which may produce complicating factors; thus, the scientific study of pain can be additionally complex.

The basic scientist attempts to correlate the antinociceptive and analgesic properties of a drug so that preliminary research on pain can be conducted in the laboratory. Here, behavioral responses to a variety of noxious stimuli in several laboratory animals are measured (see section 5.1.3). While this procedure does not appear to be valid for all drugs which produce antinociception, a correlation does exist between the antinociceptive and analgesic properties of a particular drug or group of drugs such as drugs which affect the cholinergic system (see section 2). The antinociceptive action of the anticholinesterase agent, diisopropyl phosphofluoridate (DFP), was studied in this dissertation.

Certain factors, which can interfere with the correct assessment of antinociception, are inherent in every testing procedure. For instance, here, DFP is known to depress locomotor activity and

produce hypothermia (see section 2). While these two effects of DFP were controlled during the present studies, either effect could have interfered with the correct evaluation of antinociception (see sections 5.3 and 5.4).

It also should be pointed out that DFP may produce antinociception by stimulating Renshaw Cells. Here, the apparent antinociception would be attributable to a decreased alpha motor neuron excitability, thus, a decreased ability to respond. This mechanism, however, seems unlikely since cholinergic-induced antinociception is known to arise from stimulation of supraspinal cholinergic receptors (see section 1.2.3.2).

The ultimate test, however, is to determine whether the drug in question produces a clinically significant level of analgesia when administered to humans. The clinical value must take into account potential deleterious side effects as well as the effective level of analgesia produced by the drug. Thus, pain studies can be conducted only in the clinical setting.

While DFP is too toxic for use in clinical studies, another anticholinesterase agent, physostigmine, is available and approved for clinical use. A study to determine the effect of physostigmine on the pain state in narcotic-tolerant individuals is described in the appendix (see appendix 1).

## 6. SUMMARY

Diisopropyl phosphofluoridate (DFP; 0.1 to 1.5 mg/kg s.c.) produced antinociception in rats as measured by the hot plate test. The duration and intensity of the antinociceptive response increased as the dose of DFP was increased. Administered 30 minutes after the administration of DFP (1.5 mg/kg s.c.), atropine sulfate (4.0 mg/kg i.p.) antagonized while atropine methyl nitrate (4.0 mg/kg i.p.) did not affect the DFP-induced antinociceptive response. Administered in doses of 1.0 and 1.5 mg/kg s.c., DFP produced antinociception in rats as measured by the tail flick test. DFP (0.1 to 1.5 mg/kg s.c.) decreased exploratory and motor activity levels in rats. Administered in a dose of 1.0 mg/kg s.c., DFP decreased rectal temperatures in rats. Atropine sulfate (4.0 mg/kg i.p.) failed to alter the diminution in exploration or motor activity produced by DFP. These results indicate that DFP-induced antinociception in rats arises via actions involving central cholinergic system; antinociception produced by DFP was independent of the locomotor depression and hypothermia produced by DFP.

DFP (1.5 mg/kg s.c.) increased serotonin (5-HT) levels in rat medulla, meso-diencephalon, and telencephalon. As the serotonergic and cholinergic systems have been implicated in antinociception, DFP-induced antinociception could be attributed to either system; parachlorophenylalanine (PCPA) was employed to clarify this point. Administered alone, PCPA (i) decreased 5-HT levels in all three rat

brain regions studied, (ii) produced hypernociception as measured by the hot plate and tail flick tests, and (iii) increased motor activity levels; PCPA, alone, did not affect exploratory activity. Pretreatment with PCPA prevented the augmentation of 5-HT levels produced by DFP (1.5 mg/kg s.c.) but did not affect the antinociceptive state or attenuation of exploratory or motor activity levels produced by DFP. Thus, DFP-induced antinociception is independent of the serotonergic system.

Administered 30 minutes after the administration of DFP (1.0 mg/kg s.c.), naloxone (0.5 to 5.0 mg/kg i.p.) antagonized DFP-induced antinociception. MR 2266 (-5,9  $\alpha$ -diethyl-2-(3-furymethyl)-2'-hydroxy-6,7-benzomorphan, 1.0 mg/kg i.p.) and GPA 1843 (-2-allyl-2'-hydroxy-9  $\beta$ -methyl-5-phenyl-6,7-benzomorphan, 5.0 mg/kg i.p.), the active (-) isomers of narcotic antagonists, reduced morphine- and DFP-induced antinociception; MR 2267 and GPA 1847, the inactive (+) isomers of the corresponding narcotic antagonists, did not affect morphine- or DFP-induced antinociception. MR 2266, MR 2267, GPA 1843, and GPA 1847 did not produce antinociception in naive animals. Animals rendered tolerant to the antinociceptive action of morphine (3.0 mg/kg s.c.) by repeated daily injections of morphine failed to exhibit cross tolerance to an equiactive antinociceptive dose of DFP (1.0 mg/kg s.c.) or pilocarpine (2.5 mg/kg s.c.). These results suggest that DFP-induced antinociception is mediated via stereospecific opiate receptors, possibly by the release of endogenous opioid peptides. This latter phenomenon could not be demonstrated at this time.

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## APPENDIX 1

The appended clinical investigation protocol entitled  
"Effect of physostigmine on narcotic-induced analgesia" has been  
submitted to and approved by the following authorities:

Alexander G. Karczmar M.D., Ph.D. (Primary Investigator)  
Professor and Chairman  
Department of Pharmacology  
Loyola University Stritch School of Medicine  
Submitted: 5 January 1979                      Approved: 7 January 1979

Ketty Badrinath M.D. (Primary Investigator)  
Chief  
Section of Oncology  
Department of Medicine  
Loyola University Stritch School of Medicine  
Submitted: 13 January 1979                      Approved: 5 February 1979

John R. Tobin, M.D.  
Chairman  
Department of Medicine  
Loyola University Stritch School of Medicine  
Submitted: 6 February 1979                      Approved: 5 March 1979

Institutional Review Board (IRB)  
Loyola University Stritch School of Medicine  
Submitted: 7 March 1979                      Approved: 24 April 1979  
IRB Number assigned: #3/79-5b

Dept. of Health, Education and Welfare  
Public Health Service  
Food and Drug Administration  
Submitted: 10 May 1979                      Approved: 20 June 1979  
Investigational Exemption for a New Drug (IND) Number assigned: 16,412

## CLINICAL INVESTIGATION PROTOCOL

1. Introduction

Acetylcholine, various cholinomimetics, and several anticholinesterase agents exhibit an antinociceptive action and the ability to potentiate narcotic-induced antinociception in experimental animals (Chen, 1958; Harris et al., 1968; Pedigo et al., 1975). Several investigations have demonstrated that the anticholinesterase agent, physostigmine has similar actions in humans (Slaughter, 1950; Karczmar, 1977). Recent animal studies in these laboratories are particularly pertinent (Koehn and Karczmar, 1978). First, we confirmed the antinociceptive potency of anticholinesterase compounds described by others; in fact, some of those compounds appeared, in animals, to be several times more potent than morphine. Second, we have showed that this antinociceptive effect can be obtained in full in animals rendered tolerant to morphine. Presently, intractable pain is treated by the chronic administrations of narcotic analgesic compounds. Side effects associated with this treatment include nausea, drowsiness, constipation, hypotension, and increased intracranial pressure, as well as addiction liability. What is most important, there is development of tolerance to the analgetic action of the narcotic compound; thus, to achieve pain control larger and larger doses of narcotic is needed. Ultimately, the patients become refractory, as there is no dose of the narcotic that will produce analgesia short of causing respiratory depression and death.

This study is designed to test the analgetic property of physostigmine, when used in combination with narcotic analgesics. It is hoped that the results will demonstrate that it is possible to increase the pain threshold beyond that obtained with any dose of the narcotic used alone, thus, allowing the dose of narcotic analgesic to be reduced; smaller dose of the narcotic may be sufficient to produce pain control in the presence of physostigmine. Altogether, it is hoped to establish new therapeutic regimen for treating intractable as well as other associated pain states.

The feasibility and safety of the proposed study can be readily substantiated. In the past, neostigmine was used in man to produce analgesia and to potentiate analgesia produced by codeine (for references cf. Karczmar, 1977); as is well known, neostigmine is used safely for many years in myasthenic patients. Physostigmine was tested as an analgesic in normal volunteers (Sitaram et al., 1977); furthermore, it was employed in man in a number of other conditions. For instance, it is used to antagonize toxicity due to scopolamine and atropine, phenothiazines and tricyclic antidepressants; in fact, it is the treatment of choice in this latter condition (Snyder et al., 1974). It was used also in facilitating recovery from general anesthesia

(Bidwai et al., 1976; Brebner and Hadley, 1976). Furthermore, it is used currently in man to control mania, Huntington's chorea, and tardive dyskinesia, and to improve memory (for references, cf. Karczmar, 1976; 1977; Karczmar and Dun, 1978). In these various conditions, physostigmine was used in intravenous (IV), intramuscular (IM), and oral (PO) doses of up to 2-4 mg total dose; there is no report of serious side actions and none of toxicities. In some patients, nausea and abdominal cramps were noticed. More rarely, sweating and salivation were observed. However, pretreatment with a synthetic anticholinergic agent Robinul (0.15 mg IM) 30 minutes prior to the administration of physostigmine (0.5 mg IV) prevents these peripheral side effects. No further side actions or toxicities will be expected from the dose used. There are special considerations pertinent in the context of the safety of study in question. First, physostigmine is a so-called reversible inhibitor of cholinesterase, the inhibition being short-lived and not extending, in both animal and human studies, beyond 1 to 2 hours. Second, physostigmine acts via temporary accumulation of acetylcholine; as the latter is hydrolyzed upon the termination (reversal) of anticholinesterase action of physostigmine, no potentially deleterious delayed effects and no pathology result from the action of physostigmine. Third, there are specific, fast acting and safe antagonists of physostigmine; the quaternary anticholinergics such as methanthaline and propanthaline can be used to control the peripheral effects of physostigmine, such as gastrointestinal hypermotility, while atropine and scopolamine may be used to prevent both central and peripheral actions of physostigmine. Finally, physostigmine (Antilirium) is obtainable in a preparation designed for human use.

## 2. Specific Aims

This investigation is designed to establish the following:

- (A) Physostigmine potentiation of narcotic-induced analgesia in terms of:
  - (1) Latency to onset of analgesia after drug administration
  - (2) Duration of analgesic effect
  - (3) Peak analgetic action
- (B) Therapeutic evaluation of combined narcotic-physostigmine drug treatment on the subjective pain experience.
  - (1) Sensory qualities in terms of temporal, spatial, pressure et al.
  - (2) Affective qualities in terms of tension, fear, and autonomic properties.
  - (3) Evaluative qualities that describe the subjective overall intensity of the total pain experience.

## 3. Experimental Protocol

All patients and controls used in this study will be obtained by

their consent from the service of Dr. K. Badrinath, Chief, Oncology Section, Loyola University Medical Center. Patients who are presently being administered narcotic analgesics on a chronic basis for relief of intractable pain will be selected for this study. Patients shall have no known history or evidence indicating the presence of cardiac arrhythmias, demonstrable intracranial lesions or any other physical or psychological abnormalities which would by the discretion of the attending physician place them at any particular risk. The age range of the patients will vary; primarily adults (i.e. greater than 21 years of age) will be used. No financial compensation is planned as part of this study. No additional laboratory or diagnostic procedures will be employed in or solely for the purpose of this study.

#### 4. Clinical Protocol

Patients who receive narcotic analgesics on a regular basis will be utilized in this study. All patients participating in this study will be required to complete an informed consent (see pp.203). Having obtained the patients consent, the research investigator along with the patient will complete the McGill Pain Assessment Questionnaire which is used to gather a medical history as well as to initially evaluate the patients' pain status (Melzack, 1975). This requires approximately 30 minutes of the patients time.

On the test day (day 1, Table 1) a patient will receive his/her regularly scheduled pre-determined dose of narcotic analgesic. Robinul (0.15 mg IM) will be administered 30 minutes before the subsequent drug administration for the relief of pain. At that time, the narcotic analgesic physostigmine or narcotic-analgesic placebo combination will be employed in place of the regularly scheduled dose of narcotic analgesic. The correct evaluation of any drug effect requires that a double blind study be utilized. The choice of the combination for the particular patients will be randomized according to the methods described by Sokal and Rohlf (1973). Furthermore, neither the patients nor the physician administering the drugs will know whether he/she is given the narcotic analgesic-placebo or the narcotic analgesic-physostigmine combination. The patients will be asked to evaluate and compare his/her conditions for the time periods corresponding to the two drug administrations mentioned previously; all evaluations will be made by completing the McGill-Melzack Pain Questionnaire 20 minutes after the administration of the regularly scheduled dose of the narcotic analgesic and subsequent narcotic analgesic-physostigmine/placebo combination (Melzack, 1975). This procedure will be repeated (day 3, Table 1). Those patients who received the narcotic-analgesic physostigmine drug combination on day 1 will now, day 3 receive the narcotic analgesic-placebo drug combination and vice versa. The McGill-Melzack Pain Questionnaire will be administered as before. Double blind study technique will be employed. In accordance with the I.R.B.

guidelines, patient confidentiality will be secured. Patients will be identified by a coded patient identification number on all forms.

## 5. Interpretation of Data

This investigation will be evaluated by tabulating the patients responses from the McGill-Melzack Pain Questionnaire (Melzack, 1975). The original McGill-Melzack Pain Questionnaire was specifically designed to provide quantitative measures of clinical pain that can be treated statistically. The 3 major measures are: (1) the pain rating index, based on two types of numerical values that can be assigned to each word descriptor, (2) the number of words chosen, and (3) the present pain intensity based on a 1 to 5 intensity scale. Modification of the original questionnaire (i.e. the addition of part 5) was instituted to gather additional pertinent information for this study (Melzack, 1975).

Statistical analysis to determine the effectiveness of this procedure can be performed by comparing the patients responses in the various categories for the regularly scheduled dose of the narcotic analgesic and the narcotic analgesic-physostigmine/placebo combination testing sessions. If a patient's responses for the drug combination are greater than for the regularly scheduled dose of narcotic, it is rated +; if less, -; if no change, '0'. Then, a simple sign test for significance can be carried out. Similarly, the t test can be used in which the mean net changes are calculated and the differences from the mean are calculated for each testing session to determine whether a statistically significant difference has occurred. Both types of tests will be employed in this study. It is anticipated that this study will employ 20 to 40 patients. However, a pilot study utilizing 5 patients will be performed in an unblinded manner which will allow the investigators to determine the degree of significance, thus allowing for a better estimate of the number of patients (n) required. 'n' will be set on the basis of fewest patients which would be likely to show a statistically significant difference ( $p < 0.05$ ).

## 6. Risks and Potential Benefits

Risks to the patient include the potential side effects of Robinul and physostigmine treatments; dry mouth, nausea, bradycardia, and hypotension. Gastric symptoms and heart signs if they appear will be treated with clozapine and atropine respectively. Any complaints of pain at any time during the study will be recorded and a narcotic analgesic given immediately. The only actual discomfort associated with this experiment is due to the intramuscular (IM) and intravenous (IV) administrations of Robinul and physostigmine/placebo respectively.

The potential benefits of this experiment are as follows: physio-

stigmine is expected to potentiate narcotic-induced analgesia by producing a greater level of analgesia; thus, (1) the refractory patient would be responsive to the analgetic effect of the combined drug treatment and (2) the dose of the narcotic analgesic could be reduced, thus reducing the severity of associated side effects. If the results are positive this would lend evidence and support for establishing a new more effective treatment plan for the control of pain. This could also lend further evidence for the use of anticholinesterase agents as analgesic substances (new use).

## 7. Table 1

Informed consent form for each patient utilized in this study must be completed (see pp. 203).

McGill Pain Assessment Questionnaire completed before test day (Melzack, 1975).

### Test Day      Drug Administrations

Regularly scheduled, predetermined dose of narcotic analgesics administered throughout study (Narc.).

Narc.

\*McGill-Melzack Pain Questionnaire 20 min. post drug administration.

Robinul (0.15 mg IM) 30 minutes prior to Narc. + Physostigmine/Placebo

1      Narc. + Physostigmine (0.5 mg IV)/Placebo

\*McGill-Melzack Pain Questionnaire 20 min. post drug administration

Narc.

---

Narc.

2      Narc.

Narc.

---

Narc.

\*McGill-Melzack Pain Questionnaire 20 min. post drug administration

Robinul (0.15 mg IM) 30 minutes prior to Narc. + Physostigmine/Placebo



3      Narc. + Physostigmine (0.5 mg IV)/Placebo

        \*McGill-Melzack Pain Questionnaire 20 min. post drug  
        administration

Narc.

        \*McGill-Melzack Pain Questionnaire (Melzack, 1975).

8. Informed Consent

IRB Number \_\_\_\_\_

Loyola University Medical Center  
Maywood, Illinois  
Department of Medicine  
Section of Oncology and Pharmacology

Patient's Name : \_\_\_\_\_ Date: \_\_\_\_\_

Project Title: \_\_\_\_\_

8.1 Patient Information8.1.1 Description and Explanation of Procedure

Your illness requires frequent and repeated administration of narcotic analgesic drugs to prevent the pain which you experience. As you become tolerant to the analgetic action of the narcotics the dose must be increased to remain effective. Associated with the chronic administration of narcotics are several side effects; most notably nausea, drowsiness, constipation, and respiratory depression. Sometimes your pain cannot be controlled satisfactorily even as we increase the dose. The purpose of this investigation is to study the usefulness of the drug physostigmine, used in a combination therapy program with the narcotic analgesic to potentiate the effective level of analgesia. This would allow the dose of the narcotic to be reduced lessening the severity of side effects attributable to narcotic administration, and may improve pain control and your well-being.

The correct pharmacological analysis requires that this study be done according to double-blind technique. To correctly evaluate the usefulness of physostigmine each patient will receive the narcotic analgesic-physostigmine combination and the narcotic analgesic-placebo (inactive compound) combination on test days 1 and 3 or vice versa. Neither the patient nor the physician administering the drug will know who receives what. In addition, 30 minutes prior to the administration of the narcotic analgesic-physostigmine/placebo combination you will receive an intramuscular (IM) injection of Robinul to prevent the appearance of side effects attributable to the administration of physostigmine. Each patient will participate in this study on three days. During this time, you will be asked to complete with the help of a research investigator five questionnaires as follows. First, the McGill Pain Assessment Questionnaire will be administered on the day preceding the first test day. This will require approximately 30 minutes of your time. Second, the McGill-Melzack Pain Questionnaire will be administered two times each on test days 1 and 3. You will be asked to complete this form following your regularly scheduled dose of narcotic analgesic as well as following the combination regularly scheduled dose of narcotic analgesic with physostigmine/placebo on each

test day (total of 4 times). No participation is required on test day 2.

#### 8.1.2 Risks and Discomforts

There are essentially no life risks involved using physostigmine at this dose (0.5 mg); potential adverse side effects are few. Some patients report feelings of nausea which can be remedied by administration of Clozapine or Compazine. Bradycardia (slow heart) constitutes another side effect which has developed in some patients. Your heart rate and blood pressure will be monitored and in need can be corrected by administration of atropine. The only discomfort associated with this procedure would be due to the needle for intramuscular (IM) injection of Robinul and the intravenous (IV) injection of physostigmine/placebo.

I understand that biomedical or behavioral research such as that in which I have agreed to participate, by its nature, involves risk of injury. In the event of physical injury resulting from these research procedures, emergency medical treatment will be provided at no cost, in accordance with the policy of Loyola University Medical Center. No additional free medical treatment or compensation will be provided except as required by Illinois law.

In the event you believe that you have suffered any physical injury as the result of participation in the research program, please contact Dr. H.J. Blumenthal, Chairman, Institutional Review Board for Protection of Human Subjects at the Medical Center, telephone (312) 531-3384.

#### 8.1.3 Potential Benefits

The benefit to you that we hope for will be a lessening of pain, and reduction of untoward side effects due to narcotic drug administration (drowsiness and constipation). We also hope that this particular therapeutic regimen will prove to be effective and thus establish a new procedure for treating patients who require this type of care.

#### 8.1.4 Alternatives

The alternative method for management of your pain is to be maintained on a narcotic analgesic treatment plan. The side effects and risks associated with this therapy are stated previously.

Consent

I have fully explained to \_\_\_\_\_ (name: patient) \_\_\_\_\_ the nature and purpose of the above described procedure and the risks that are involved in its performance. I have answered and will answer all questions to the best of my ability.

\_\_\_\_\_  
(signature: principal investigator)

I have been fully informed of the above-described procedure with its possible benefits and risks. I give permission for my participation in this study. I know that Dr. Ketty Badrinath or his associates will be available to answer any questions I may have. If, at any time, I feel my questions have not been adequately answered, I may request to speak with a member of the Medical Center Institutional Review Board. I understand that I am free to withdraw this consent and discontinue participation in this project at any time without prejudice to my medical care. I have received a copy of this informed consent document.

\_\_\_\_\_  
(signature: patient)

\_\_\_\_\_  
(signature: witness to signatures)

APPROVAL SHEET

The dissertation submitted by Gary L. Koehn has been read and approved by the following committee:

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Date

5/2/1980

Director's Signature

